EINO OLAVI ERÄNKÖ
Department of Anatomy
University of Helsinki
Siltavuorenpenger, Helsinki, Finland

STUDIES ON THE SYMPATHICO-ADRENAL SYSTEM

Published work submitted for the Degree
of D Sc in the University of Edinburgh

Helsinki

May 1974

CONTENTS

9. O. Eränkö, A. Kokko & U. Söderholm: Histochemical evidence of three types of esterases in the adrenal medulla of the rat. Histochemie 1962, 2, 383-388.

10. O. Eränkö & V. Hopsu: Effect of denervation on reserpine-induced depletion and subsequent restitution of adrenaline and noradrenaline in the adrenal medulla of the rat. Ann. Med. Exper. Fenn. 1962, 40, 134-145.

11. O. Eränkö & M. Härkönen: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. Acta physiol. scand. 1963, 58, 285-286.

12. O. Eränkö: Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. J. Histochem. Cytochem. 1964, 12, 487-488.

13. O. Eränkö & M. Härkönen: Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. Acta physiol. scand. 1964, 61, 299-300.

14. O. Eränkö, M. Härkönen, A. Kokko & L. Räisänen: Histochemical and starch gel electrophoretic characterization of desmo- and lyo-esterases in the sympathetic and spinal ganglia of the rat. J. Histochem. Cytochem. 1964, 12, 570-581.

15. O. Eränkö & M. Härkönen: Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. Acta physiol. scand. 1965, 63, 411-412.

16. O. Eränkö & L. Räisänen: Fibres containing both noradrenaline and acetylcholinesterase in the nerve net of the rat iris. Acta physiol. scand. 1965, 63, 505-506.

17. O. Eränkö & M. Härkönen: Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. Acta physiol. scand. 1965, 63, 511-512.

18. O. Eränkö & L. Räisänen: In vitro release and uptake of noradrenaline in the rat iris. In "Mechanisms of Release of Biogenic Amines" (Eds. U.S. von Euler, S. Rosell and B. Uvnäs), Pergamon Press, Stockholm 1966, 73-78.

19. O. Eränkö, M. Lempinen & L. Räisänen: Adrenaline and noradrenaline in the organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. Acta physiol. scand. 1966, 66, 253-254.

20. O. Eränkö: Demonstration of catecholamines and cholinesterases in the same section. Pharmacol. Revs. 1966, 18, 353-358.

21. O. Eränkö & L. Räisänen: Demonstration of catecholamines in adrenergic nerve fibres by fixation in aqueous formaldehyde solution and fluorescence microscopy. J. Histochem. Cytochem. 1966, 14, 690-691.

22. O. Eränkö: Histochemistry of nervous tissues: catecholamines and cholinesterases. Ann. Rev. Pharmacol. 1967, 7, 203-222.

23. O. Eränkö, L. Rechardt & L. Hänninen: Electron microscopic demonstration of cholinesterases in nervous tissue. Histochemie 1967, 8, 369-376.

24. O. Eränkö, M. Lempinen & L. Räisänen: Effect of hydrocortisone administration in utero on the adrenaline and noradrenaline content of extra-adrenal chromaffin tissue in the rat. Acta physiol. scand. 1967, 69, 255-256.

25. G. Åberg & O. Eränkö: Localization of noradrenaline and acetylcholinesterase in the taenia of the guinea-pig caecum. Acta physiol. scand. 1967, 69, 383-384.

26. O. Eränkö: The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. J. Roy. Micr. Soc. 1967, 87, 259-276.

27. O. Eränkö & H. Teräväinen: Distribution of esterases in the myoneural junction of the striated muscle of the rat. J. Histochem. Cytochem. 1967, 15, 399-403.

28. O. Eränkö & H. Teräväinen: Cholinesterases and eserine-resistant carboxylic esterases in degenerating and regenerating motor end plates of the rat. J. Neurochem. 1967, 14, 947-954.

29. O. Eränkö, L. Rechardt, L. Eränkö & A. Cunningham: Light and electron microscopic histochemical observations on cholinesterase-containing sympathetic nerve fibres in the pineal body of the rat. Histochem. J. 1970, 2, 479-489.

30. O. Eränkö & L. Eränkö: Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. In Histochemistry of Nervous Transmission (Ed. O. Eränkö), Progress in Brain Research, Elsevier, Amsterdam, London, New York, 1971, 34, 39-51.

31. O. Eränkö & L. Eränkö: Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of the pineal body of the rat after chemical sympathectomy with 6-hydroxydopamine. Histochem. J. 1971, 3, 357-363.

32. O. Eränkö & L. Eränkö: Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats. Histochem. J. 1971, 3, 451-456.

33. L. Eränkö & O. Eränkö: Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats. Acta pharmacol.toxicol. 1971, 30, 403-416.

34. L. Eränkö & O. Eränkö: Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat. Acta physiol. scand. 1972, 84, 115-124.

35. L. Eränkö & O. Eränkö: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. Acta physiol. scand. 1972, 84, 125-133.

36. O. Eränkö, L. Eränkö, C.E. Hill & G. Burnstock: Hydrocortisone-induced increase in the number of small intensely fluorescent cells and their histochemically demonstrable catecholamine content in cultures of sympathetic ganglia of the newborn rat. Histochem. J. 1972, 4, 49-58.

37. O. Eränkö: Light and electron microscopic histochemical evidence of granular and non-granular storage of catecholamines in the sympathetic ganglion of the rat. Histochem. J. 1972, 4, 213-224.

38. L. Eränkö, C. Hill, O. Eränkö & G. Burnstock: Lack of toxic effect of guanethidine on nerve cells and small intensely fluorescent cells in cultures of sympathetic ganglia of newborn rats. Brain Res. 1972, 43, 501-513.

39. O. Eränkö, J. Heath & L. Eränkö: Effect of hydrocortisone on the ultrastructure of the small, intensely fluorescent granule-containing cells in cultures of sympathetic ganglia of newborn rats. Z. Zellforsch. 1972, 134, 297-310.

40. O. Eränkö, J. Heath & L. Eränkö: Effect of hydrocortisone on the ultrastructure of the small, granule-containing cells in the superior cervical ganglion of the newborn rat. Experientia 1973, 29, 457-459.

41. J. Heath, O. Eränkö & L. Eränkö: Effect of guanethidine on the ultrastructure of the small granule-containing cells in cultures of rat sympathetic ganglia. Acta pharmacol. toxicol. 1973, 33, 209-218.

42. C.E. Hill, G.E. Mark, O. Eränkö, L. Eränkö & G. Burnstock: Use of tissue culture to examine the actions of guanethidine and 6-hydroxydopamine. Eur. J. Pharmacol. 1973, 23, 162-174.

43. O. Korkala, O. Eränkö, S. Partanen, L. Eränkö & A. Hervonen: Histochemically demonstrable increase in the catecholamine content of the carotid body in adult rats treated with methylprednisolone or hydrocortisone. Histochem. J. 1973, 5, 479-485.

44. M. Costa, L. Eränkö & O. Eränkö: Effect of hydrocortisone on the extra-adrenal intensely fluorescent chromaffin and non-chromaffin cells in stretch preparations of the newborn rat. Z. Zellforsch. 1973, 146, 565-578.

45. M. Costa & O. Eränkö: Histochemical correlates of cold-induced trans-synaptic induction in the rat superior cervical ganglion. Histochem. J. 1974, 6, 35-53.

46. M. Costa, O. Eränkö & L. Eränkö: Hydrocortisone-induced increase in the histochemically demonstrable catecholamine content of sympathetic neurons of the newborn rat. Brain Res. 1974, 67, 457-466.

Histochemie, Bd. 1, S. 257—267 (1959)

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki

SPECIFIC DEMONSTRATION OF ACETYLCHOLINESTERASE AND NON-SPECIFIC CHOLINESTERASE IN THE ADRENAL GLAND OF THE RAT*

By

OLAVI ERÄNKÖ

With 8 Figures in the Text, of which 2 in Colour

(Eingegangen am 5. Februar 1959)

It is generally agreed upon that the secretory nerve impulses reach the adrenal medulla through preganglionic sympathetic nerve fibers (HOLLINSHEAD 1936, HOLLINSHEAD and FINKELSTEIN 1937, YOUNG 1939) and that acetylcholine plays an important role in the transmission of the impulses to the parenchymal cells (FELDBERG et al. 1934). Cholinesterase, which breaks down acetylcholine, is known to be present in the adrenal medulla (ANTOPOL and GLICK 1940, ORD and THOMPSON 1950, and others, see CHESSICK 1954). KOELLE (1950) was the first to demonstrate cholinesterase histochemically; he observed acetylcholinesterase exclusively in the nerve fibers of the adrenal medulla but non-specific cholinesterase also in the parenchymal cells. With an improved technique, KOELLE (1951), however, found cholinesterase exclusively in the nervous structures of the adrenal medulla and concluded that the previously observed reaction in the chromaffin cells presumably resulted from diffusion from the preganglionic fibers and plasma.

KOELLE (1951) used DFP (di-*iso*propylfluorophosphate) for the inhibition of non-specific cholinesterase, and this inhibitor has since then been extensively used in histochemical studies for the demonstration of acetylcholinesterase, amongst others by COUPLAND and HOLMES (1958) in a study on adrenal cholinesterases. However, several investigations suggest that acetylthiocholine may be split by enzymes other than acetylcholinesterase even when DFP is used as an inhibitor, and that butyrylthiocholine may be decomposed by enzymes other than non-specific cholinesterase (ORD and THOMPSON 1952, CHESSICK 1954, KOELLE 1955, BAYLISS and TODRICK 1956, PEPLER and PEARSE 1957, ALLEN, ERÄNKÖ and HUNTER 1958). BAYLISS and TODRICK (1956) suggested that the biochemical determination of acetylcholinesterase in the presence of excess non-specific cholinesterase, or vice versa, could be improved by using selective inhibitors. PEPLER and PEARSE (1957) applied the same principle to histochemical studies. For inhibiting specifically acetylcholinesterase they used 62. C. 47 (1:5-bis-(4-trimethylammonium-phenyl)pentan-3-one di-iodide) (BURGEN 1949), and as a selective inhibitor of non-specific cholinesterase *iso*-OMPA (tetra*iso*propyl-pyrophosphoramide) (ALDRIGE 1953), with excellent results.

In a previous study (ALLEN, ERÄNKÖ and HUNTER 1957, 1958), in which acetylthiocholine, butyrylthiocholine, myristoyl choline and α-naphtyl acetate

* This work has been supported by a research grant from the National Institute of Arthritis and Metabolic Diseases, the U.S. Public Health Service (A-1725) and by a grant from Finland's Cultural Fund.

were used as substrates, and eserine, DFP, Ro 2—0683 ((2-hydroxy-5-phenyl-benzyl)-trimethylammonium bromide) and Ro 2—1250 (p-chlorphenyl-methyl carbonate of *m*-dimethylaminophenol-methyl bromide) as inhibitors, it was observed that the reactions obtained with the different substrates employed were characterized by different inhibition patterns. The results can be explained by assuming that there are several enzymes with overlapping properties or that both acetylcholinesterase and non-specific cholinesterase have different activities towards different substrates, neither being selectively inhibited by the inhibitors used but to a variable degree inhibited by all of them. The present work was designed to study this problem by using 62. C. 47 and *iso*-OMPA as selective inhibitors. The subject has been briefly treated in a preliminary communication (ERÄNKÖ 1958).

Materials and Methods

Adult healthy rats of the Wistar strain were used. The animals were killed by decapitation with sharp scissors without preceding anaesthesia. The adrenals were dissected free of surrounding adipose tissue and either plunged in the fixative as a whole or frozen fresh on the tissue holder of a freezing microtome.

Sections cut after 2—3 hour's fixation in calcium formol (1 volume of concentrated analytical formalin solution, 3 volumes of distilled water and 6 volumes of 2 per cent calcium chloride) were rinsed in distilled water, floated on slides and allowed to dry. Fresh-frozen sections were either immersed directly in the histochemical reagents, allowed to dry unfixed on clean slides, or fixed for 2—5 min in the calcium-formol solution, rinsed in water and floated on slides. The section thickness varied from 10 to 50 micra. The distributions of the cholinesterase reactions were similar in the fresh unfixed sections and in the calcium-formol-fixed sections. Therefore, no further attention will be paid to this matter in the description of the results.

The fluorescent islets of the adrenal medulla (ERÄNKÖ 1951, 1952), composed of noradrenaline-containing parenchymal cells (ERÄNKÖ 1955), were demonstrated by fluorescence microscopy in the sections cut from the adrenals fixed in calcium formol. The fluorescence was recorded photographically and the same sections were then treated for the demonstration of cholinesterases.

Cholinesterase activity was studied by using the following substrates:

a) Acetyl- and butyrylthiocholine. The improved method described by KOELLE (1951) was used with the omission of storage solutions. Attempts were made to simplify the method by omitting sodium sulfate from the substrate mixture and copper thiocholine from the rinsing baths, with disappointing results. Therefore, the prescribed method was later strictly followed.

The inhibitors (eserine, DFP, 62. C. 47 and *iso*-OMPA) were dissolved both in a pre-incubation mixture, identical of composition with the substrate mixture except for the omission of the substrate, and in the substrate mixture. The sections were incubated in the pre-incubation mixture for 20—30 min at 37° C and subsequently in the substrate solution for 1—4 hours at the same temperature.

b) α-Naphtyl acetate. The technique prescribed by PEARSE (1953) was used. The phosphate buffer was adjusted to p_H 7.4. However, the concentration of the diazonium salt was increased from 1 mg per cc, as prescribed by PEARSE, to 5—10 mg per cc, since this had earlier been shown to result in an improved localization of the enzyme activity (ALLEN, ERÄNKÖ and HUNTER 1958). As a coupling agent Brentamine Fast Red B Salt (diazotate of 5-nitro-*ortho*-anisidine) gave good results.

In the inhibitor studies with this substrate, the sections were first pre-incubated in the same inhibitor-containing pre-incubation mixtures which were used for studies with acetyl- and butyrylthiocholine, and then covered with the α-naphtyl-acetate-containing substrate mixture into which the same inhibitor was added in the same concentration. Pre-incubation was carried out for 20—30 min at 37° C, and incubation with the substrate for 15—30 min at room temperature.

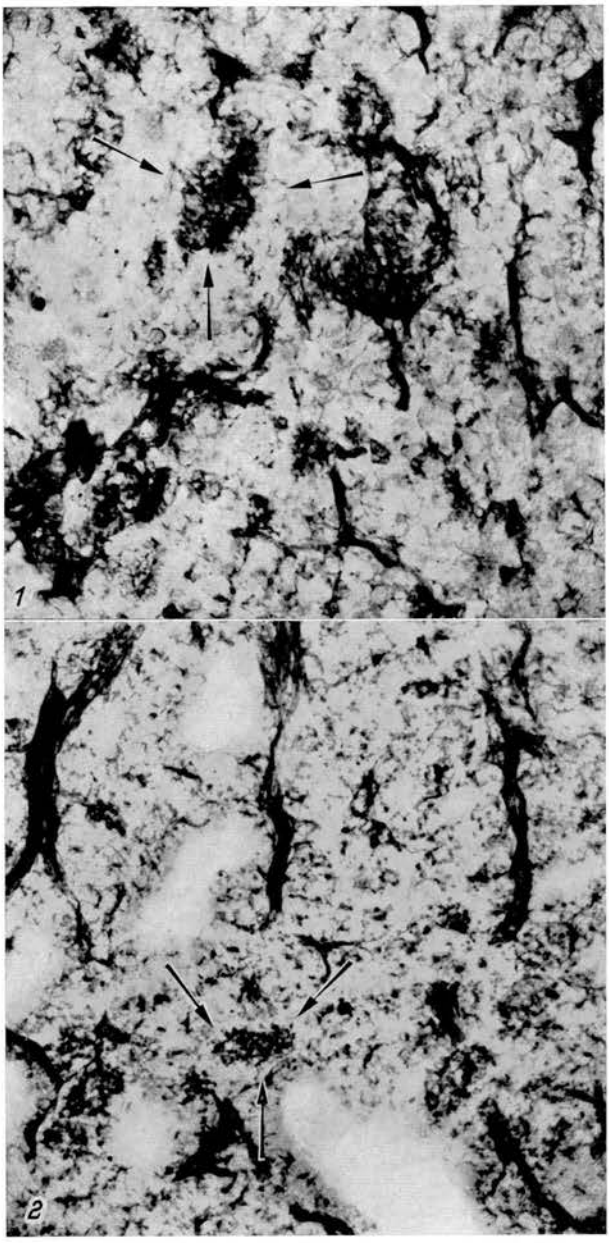


Fig. 1. Distribution of non-specific cholinesterase in the adrenal medulla. Nerve trunks, coarse nerve fibers and the fiber nets in the regions of the fluorescent cell islets (one indicated by arrows) strongly positive. Between these positive areas the fine fibers are weakly stained but the background reaction in the parenchymal cells is stronger than that in Fig. 2. Butyrylthiocholine and 62. C. 47. (Similar distribution is obtained with butyrylthiocholine alone.) Magnification ×110

Fig. 2. Distribution of acetylcholinesterase in the adrenal medulla. Coarse nerve trunks and the ovoid bodies along the fine medullary fibers are strongly positive. Regions of the fluorescent cell islets (one indicated by arrows) hardly recognizably different from the surroundings. Acetylthiocholine and *iso*-OMPA. Magnification ×110

Results

1. Acetylthiocholine. Sections incubated with acetylthiocholine in the absence of any inhibitor showed a positive reaction in the capsule, in the nerve trunks, whether in the region of the cortex or the medulla, in the small medullary ganglia and in the medullary nerve net. Areas corresponding to the fluorescent — i.e. noradrenaline-containing — medullary cell islets were strongly positive as com-

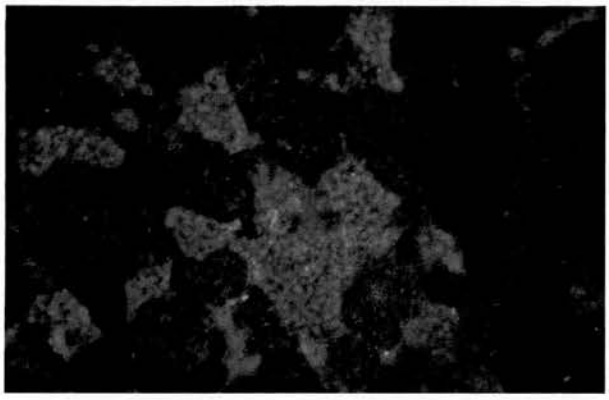


Fig. 3

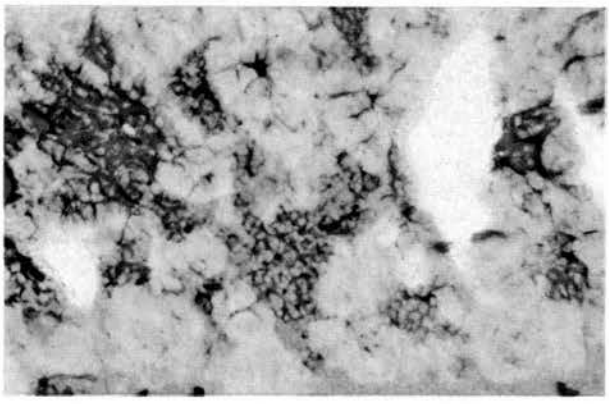


Fig. 4

Fig. 3. Fluorescence photomicrograph of adrenal medulla fixed for 3 hours in calcium formol. Fluorescent cell islets bright green, empty blood vessels dark. Magnification ×110

Fig. 4. The same section after demonstration of non-specific cholinesterase with α-naphtol acetate. Note that the reaction is limited to the regions of the fluorescent islets, with the exception of bundles of coarse nerve fibers shown above left and right

pared with the rest of the medullary parenchyma, excepting the ganglion cells and the coarse nerve fibers. Everywhere in the medulla fine fibers with small strongly positive bodies were seen. The parenchymal cells themselves were essentially negative after incubation for 1—$1^1/_2$ hours but became slightly positive after prolonged incubation.

Eserine (10^{-6} *M*) completely prevented the reaction with this substrate. Various concentrations of *iso*-OMPA were tried. Considerable weakening of the reaction all-over the gland was caused by concentrations higher than 10^{-5} *M* of *iso*-OMPA, while a concentration of 10^{-6} *M* caused only a limited reduction,

as judged without a microscope against a white background. Microscopic examination revealed, however, some differences in the appearance of the sections incubated with 10^{-6} *M* *iso*-OMPA, as compared with those incubated in the same substrate mixture but without any inhibitor. The coarse nerve trunks and the ganglion cells were still positive but the capsule had turned negative, and the nerve nets which were strongly positive in the regions of the fluorescent islets in the sections incubated without inhibitor were less prominent owing to the reduction in the intensity of the reaction by *iso*-OMPA. The network of fine fibers all-over the medullary parenchyma was therefore better visible. The small spherical or ovoid bodies at short intervals along the fibers were strongly positive (Fig. 2).

Since DFP has much been used in the purpose of inhibiting non-specific cholinesterase to demonstrate acetylcholinesterase only, sections incubated with acetylthiocholine and various concentrations of DFP were compared with sections from the same gland incubated with the same substrate but with *iso*-OMPA. It was thus observed that 10^{-8} or 10^{-9} *M* DFP had the same effect as 10^{-6} *M* *iso*-OMPA. This would seem to indicate that DFP was at the concentrations employed able to inhibit the activity of non-specific cholinesterase.

Addition of 62. C. 47 into the substrate mixture in a concentration of $10^{-5}M$, or less, caused little macroscopically visible reduction in the intensity of the reaction. However, the microscopic distribution of the reaction was different from that obtained with acetylthiocholine and *iso*-OMPA but similar to that obtained by using butyrylthiocholine, to be described next.

2. Butyrylthiocholine. The reaction obtained with this substrate in the absence of any inhibitor resembled in certain respects that obtained with acetylthiocholine without inhibitor, when short incubation times (1—$1^1/_2$ hours) were used. The capsule, the nerve trunks, the small medullary ganglions and the areas corresponding to the fluorescent parenchymal cell islets in the medulla were strongly positive (Fig. 1). The rest of the medulla was, as with acetylthiocholine and 62. C. 47, almost negative but closer examination showed a weak reaction in fine fibers, probably nerve fibers, even in these regions, and the small spherical bodies could be detected along them, although the reaction in these bodies was weak with butyrylthiocholine. The only difference in the results obtained with butyrylthiocholine and the combination of acetylthiocholine and 62. C. 47 was that the cytoplasm of the parenchymal cells became yellowish earlier than with acetylthiocholine, i.e., already after $1^1/_2$ hour's incubation in butyrylthiocholine.

While 10^{-6} *M* eserine abolished the reaction completely when butyrylthiocholine was used as a substrate, 62. C. 47 had no effect on it up to concentrations of 10^{-5} *M*. Even when a concentration of 10^{-3} *M* was used, which resulted in some weakening in the intensity of the reaction, its distribution remained the same. On the other hand, addition of *iso*-OMPA in a concentration of 10^{-6} *M* or DFP in a concentration of 10^{-8} *M*, which did not much reduce the intensity of the reaction when acetylthiocholine was used as a substrate, rendered the reaction with butyrylthiocholine entirely negative.

In further studies 10^{-6} *M* *iso*-OMPA was routinely added into the acetylthiocholine-containing substrate mixture for the demonstration of acetylcholinesterase and 10^{-5} *M* 62. C. 47 into the solution of butyrylthiocholine for the demonstration of non-specific cholinesterase. Control sections were always parallelly

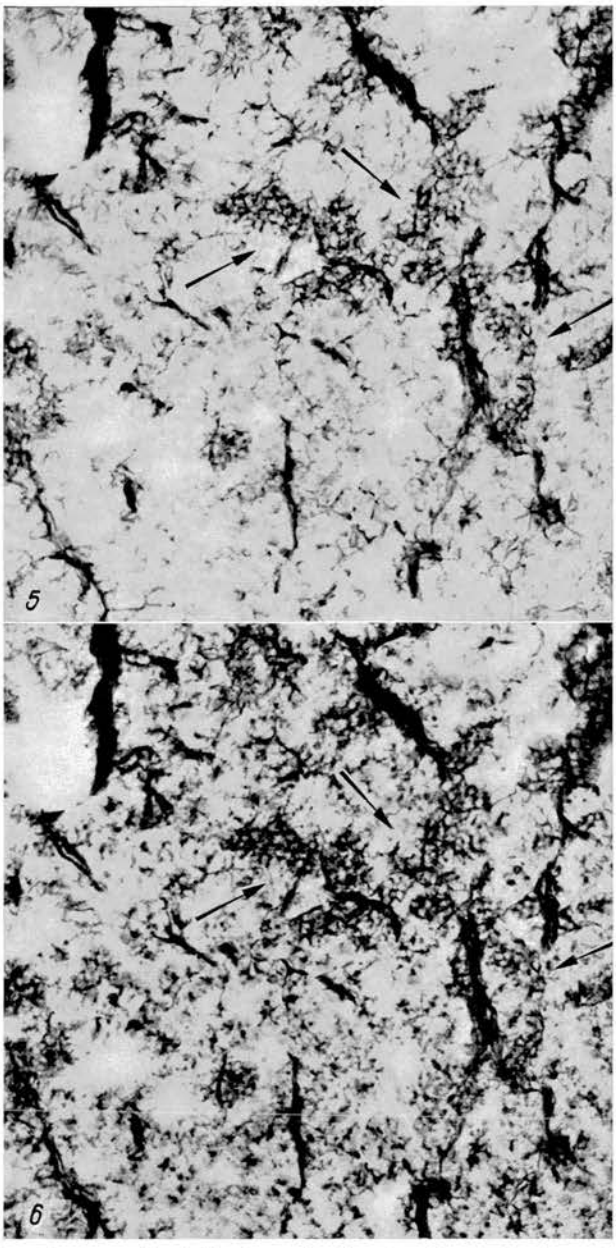


Fig. 5. Distribution of non-specific cholinesterase in the adrenal medulla, demonstrated by butyryl-thiocholine. Note the almost selective reaction in the nerve trunks and the regions of fluorescent cell islets (some indicated by arrows). Magnification ×110

Fig. 6. Same section after further treatment with acetylthiocholine and *iso*-OMPA to demonstrate acetylcholinesterase. Note the appearance of positive reaction between the nerve trunks and the formerly positive fiber nets

incubated in a mixture containing both thiocholine substrates and both 10^{-5} *M* *iso*-OMPA and 10^{-6} *M* 62. C. 47; they were found always negative.

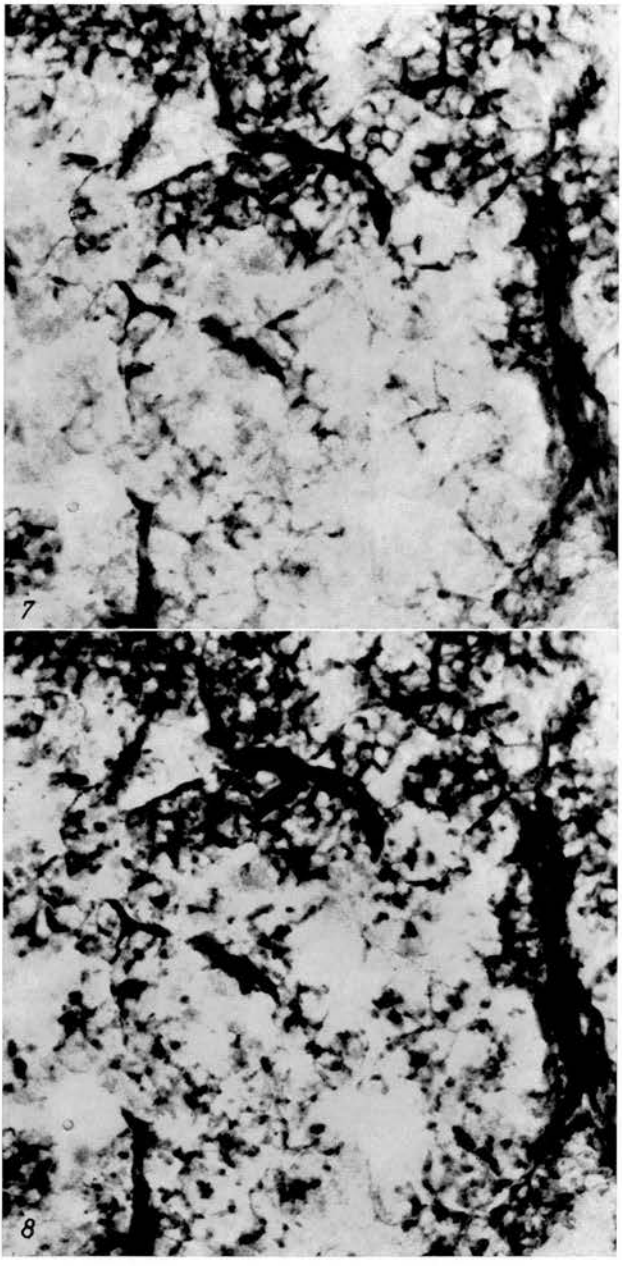


Fig. 7. A detail of Fig. 5. Magnification ×250

Fig. 8. A detail of Fig. 6. Magnification ×250

3. α-Naphtyl acetate. Results obtained with this substrate resembled in many respects those obtained with butyrylthiocholine (Figs. 3 and 4). The distribution of the reaction in the medulla was similar excepting the macrophages, which showed a very strong reaction with α-naphtyl acetate. The whole cortex was

also strongly positive with this substrate. The cortex was stained intensely with α-naphtyl acetate even in the presence of 10^{-5} *M* eserine, although the medullary structures were negative.

The eserine-sensitive part of the reaction obtained with α-naphtyl acetate showed not only a distribution similar to that obtained with butyrylthiocholine but also a similar behaviour towards 62. C. 47, DFP and *iso*-OMPA. Thus, 10^{-5} *M* 62. C. 47 had no effect on the reaction, while 10^{-6} *M* *iso*-OMPA totally and 10^{-8} *M* DFP almost totally inhibited the reaction, with the exception of the eserine-resistant cortex.

4. Comparison of acetylcholinesterase and non-specific cholinesterase in the same section. To study more carefully the difference observed in the distributions of acetyl- and non-specific cholinesterase, use was made of the fact that, under the conditions of the present study, the distribution pattern obtained with butyrylthiocholine without inhibitor was similar to that seen with the same substrate and 62. C. 47, indicating that this substrate was attacked only by non-specific cholinesterase but not by acetylcholinesterase. This rendered possible to demonstrate first non-specific cholinesterase by incubation in butyrylthiocholine and then, superimposed in the same section, acetylcholinesterase by incubation in a mixture containing acetylthiocholine and *iso*-OMPA. Comparison of pairs of photomicrographs thus made (Figs. 5—8) clearly confirmed the dominance of non-specific cholinesterase in the fiber nets around the fluorescent parenchymal cells and the dominance of acetylcholinesterase in the small bodies along the fine nerve fibers all-over the medulla, thus proving the difference in the distributions of these two enzymes. Differences were also encountered in the distributions of the enzymes in the small medullary ganglia, non-specific cholinesterase being localized predominantly in the structures around the ganglion cells, acetylcholinesterase in the cytoplasm of these cells.

Discussion

It was observed in the present study that relatively high concentrations of 62. C. 47 failed to have essential influence on the reactions obtained with butyrylthiocholine or α-naphtyl acetate, while 10^{-6} *M* *iso*-OMPA or 10^{-8} *M* DFP inhibited the cholinesterase activity demonstrable with these two substrates. (Positive reaction with α-naphtyl acetate in the cortex was eserine-resistant and thus not due to cholinesterase activity). This behaviour of butyrylthiocholine is not surprising knowing that this substance is easily hydrolyzed by non-specific cholinesterase but little or not at all by acetylcholinesterase (KOELLE 1951, 1955, ALDRIDGE 1953, PEARSE 1953).

The total inhibition by 10^{-6} *M* *iso*-OMPA and almost total inhibition by 10^{-8} *M* DFP of the eserine-sensitive component of the reaction obtained with α-naphtyl acetate is, on the other hand, of interest, because earlier work suggests that this substrate can be used for the demonstration of acetylcholinesterase, and because higher concentrations of DFP have been observed necessary to inhibit the reaction obtained with α-naphtyl acetate than that with butyrylthiocholine (PEARSE 1953, ALLEN, ERÄNKÖ and HUNTER 1958). It may be possible that factors such as the nature and concentration of the diazonium compounds used as coupling agents with α-naphtyl acetate have an influence on the inhibition

pattern. The concentration of the diazonium salts recommended by PEARSE (1953) and used by ALLEN, ERÄNKÖ and HUNTER (1958) in the inhibition studies was 1 mg per cc, while 5—10 mg/cc was used in the present study to improve the localization of the reaction. If the diazonium salt employed has stronger inhibitory effect on acetylcholinesterase than on non-specific cholinesterase, the apparent discrepancy can be understood.

Although several studies seem to indicate that cholinesterase, conventionally divided into two varieties, i.e., true or acetylcholinesterase and pseudo- or non-specific cholinesterase, really cover a group of enzymes with overlapping properties (e.g. CHESSICK 1954, KOELLE 1955, PEPLER and PEARSE 1957, ALLEN, ERÄNKÖ and HUNTER 1958), the observations of the present study are in conformity with the conventional assumption of the presence of only two types of cholinesterases.

Acetylthiocholine and *iso*-OMPA or DFP seem to provide good possibilities for the study of acetylcholinesterase without fear of interference by non-specific cholinesterase. Earlier claim by the present writer (ERÄNKÖ 1958) that differentiation of non-specific from acetylcholinesterase can be made with *iso*-OMPA but not with DFP has now been found erroneous and due to the use of DFP solutions which had lost their potency. Unless the fairly rapid deterioration of DFP solutions has been overlooked also by other workers, the earlier observations on the distribution of acetylcholinesterase made using DFP should be valid.

Butyrylthiocholine alone or acetylthiocholine with 62. C. 47 can in many cases be used to demonstrate histochemically non-specific cholinesterase without interference by acetylcholinesterase. When eserine-resistant esterases can be excluded, α-naphtyl acetate would in many cases seem to fulfil adequately the same task. However, the combination of butyrylthiocholine with 62. C. 47 is obviously safer and should be used, unless evidence is already available that high acetylcholinesterase activity does not interfere.

As to the distribution of cholinesterases in the adrenal gland, the observations of the present study are in most respects in agreement with those reported in the only two earlier studies which deal in some detail with this subject (ALLEN, ERÄNKÖ and HUNTER 1958, COUPLAND and HOLMES 1958). Since complete inhibition was used as an end point in the study by the former workers of the effect of various inhibitors, the different distributions obtained with and without DFP remained overlooked. The latter two authors, who also studied the adrenals of the rat, were careful enough to notice that the finer strands of the medullary mesh are well revealed by acetylthiocholine, but not by butyrylthiocholine, an observation confirmed in the present study with short incubation times. The present work also confirms the presence of small ovoid bodies along the fine medullary fibers described by COUPLAND and HOLMES (1958) and shows that these bodies have a particularly strong acetylcholinesterase activity.

It seems that the coarse nerve trunks and fibers and the medullary ganglia contain both non-specific and acetylcholinesterase, although the latter predominates in the cytoplasm of the ganglion cells. This is in agreement with results obtained with nervous tissue elsewhere in the body (KOELLE 1951, 1955). The adrenal capsule and the fibers associated with the noradrenaline-containing medullary cell islets exhibit a strong activity of non-specific cholinesterase,

and the fine meshwork of nerve fibers a strong acetylcholinesterase activity. While the presence of acetylcholinesterase in the nervous elements is logically easy to understand, the selective association of non-specific cholinesterase in the regions of the noradrenaline cells is somewhat surprising. Although the reaction was strongly positive in the fibers around the parenchymal cells rather than in the cells themselves, which reacted but weakly, the thought is near that non-specific cholinesterase would somehow be connected in the metabolism of the noradrenaline-containing cells. However, this problem requires further study.

Summary

Distribution of cholinesterase in the adrenal medulla of the rat was studied using acetylthiocholine, butyrylthiocholine and α-naphtyl acetate as substrates and eserine, di-*iso*propylfluorophosphate (DFP), 1:5-*bis*-(4-trimethylammonium-phenyl)-pentan-3-one di-iodide (62. C. 47) and tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) as inhibitors.

Acetylcholinesterase was observed in the nerve trunks, the ganglion cells, the coarse and the fine nerve fibers. The fine medullary network showed along the fibers small strongly positive ovoid bodies.

Non-specific cholinesterase was detected in the capsule, the nerve trunks, the coarse nerve fibers and the fibers surrounding the noradrenaline-containing, fluorescent medullary cell islets. A weak reaction was also seen in the cytoplasm of the medullary cells. The fine medullary fibers with the ovoid bodies were essentially negative.

A method was developed to demonstrate first non-specific cholinesterase and then acetylcholinesterase in the same section. The different distributions of the two cholinesterases were confirmed with this method.

Acknowledgments. Skillful technical assistance by Mrs. LIISA RÄISÄNEN is greatly appreciated.

Samples of 62. C. 47 were obtained through the courtesy of Dr. F. C. COPP, The Wellcome Research Laboratories, England, *iso*-OMPA through the courtesy of Messrs. L. Light and Co., Ltd., England, and DFP through the courtesy of Dr. CATHERINE HEBB, Institute of Animal Physiology, Cambridge, England.

Bibliography

ALDRIDGE, W. N.: The differentiation of true and pseudo-cholinesterase by organophosphorus compounds. Biochem. J. **53**, 62—67 (1953). — ALLEN, J. M., O. ERÄNKÖ and R. L. HUNTER: A cytochemical study of cholinesterase activity in the adrenal medulla of the rat. Anat. Rec. **128**, 514—515 (1957). — A histochemical study of the esterases of the adrenal medulla of the rat. Amer. J. Anat. **102**, 93—110 (1958). — ANTOPOL, W., and D. GLICK: Studies in histochemistry. XV. The histological distribution of choline esterase in the adrenal gland. J. biol. Chem. **132**, 669—673 (1940). — BAYLISS, B. J., and A. TODRICK: The use of a selective acetylcholinesterase inhibitor in the estimation of pseudocholinesterase activity in rat brain. Biochem. J. **62**, 62—67 (1956). — BURGEN, A. S. V.: The mechanism of action of anticholinesterase drugs. Brit. J. Pharmacol. **4**, 219—228 (1949). — CHESSICK, R.: The histochemical specificity of cholinesterases. J. Histochem. Cytochem. **4**, 341—346 (1954). — COUPLAND, R. E., and R. L. HOLMES: The distribution of cholinesterase in the adrenal glands of the rat, cat and rabbit. J. Physiol. (Lond.) **141**, 97—106 (1958). — ERÄNKÖ, O.: On the histochemistry of the rat adrenal medulla. Acta physiol. scand. **25**, Suppl. 89, 22—23 (1951). — On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. Acta anat. **16**, Supp. 17, 1—60 (1952). — Distribution of adrenaline and noradrenaline in the adrenal medulla. Nature (Lond.) **175**, 88 (1955). — Differential demon-

stration of acetylcholinesterase and non-specific cholinesterase in the adrenal medulla of the rat. Nature (Lond.) **182**, 183—184 (1958). — FELDBERG, W., B. MINZ and H. TZUDZIMURA: The mechanism of the nervous discharge of adrenaline. J. Physiol. (Lond.) **81**, 286—304 (1934). — HOLLINSHEAD, W. H.: The innervation of the adrenal glands. J. comp. Neurol. **64**, 449—467 (1936). — HOLLINSHEAD, W. H., and H. FINKELSTEIN: Regeneration of nerves to the adrenal gland. J. comp. Neurol. **67**, 215—220 (1937). — KOELLE, G. B.: The histochemical differentiation of types of cholinesterases and their localizations in tissues at the cat. J. Pharmacol. exp. Ther. **100**, 158—179 (1950). — The elimination of diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. J. Pharmacol. exp. Ther. **103**, 153—171 (1951). — The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. J. Pharmacol. exp. Ther. **114**, 167—184 (1955). — ORD, M. G., and R. H. S. THOMPSON: The distribution of cholinesterase types in mammalian tissues. Biochem. J. **46**, 346—352 (1950). — Pseudo-cholinesterase activity in the central nervous system. Biochem. J. **51**, 245—251 (1952). — PEARSE, A. G. E.: Histochemistry, theoretical and applied. London: J. & A. Churchill 1953. — PEPLER, W. J., and A. G. E. PEARSE: The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei. J. Neurochem. **1**, 193—202 (1957). — YOUNG, J. Z.: Partial degeneration of the nerve supply of the adrenal. A study in autonomic innervation. J. Anat. (Lond.) **73**, 540—550 (1939).

Prof. O. ERÄNKÖ, Department of Anatomy, University of Helsinki, Siltavuorenpenger 20, Helsinki, Finland

Druck der Universitätsdruckerei H. Stürtz AG., Würzburg

Journal of Neurochemistry, 1959, Vol. 4, pp. 264 to 267. Pergamon Press Ltd. Printed in Northern Ireland

EFFECT OF DENERVATION ON HISTOCHEMICALLY DEMONSTRABLE ACETYLCHOLINESTERASE AND NON-SPECIFIC CHOLINESTERASE IN THE RAT ADRENAL

O. ERÄNKÖ, V. HOPSU and L. RÄISÄNEN
Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki

(*Received* 24 *February* 1959)

PREVIOUS studies have indicated that the adrenal medulla possesses an intense cholinesterase activity (ANTOPOL and GLICK, 1940; ORD and THOMPSON, 1952; KOELLE, 1950, 1951; ALLEN, ERÄNKÖ and HUNTER, 1958; COUPLAND and HOLMES, 1958). This is indeed what can be expected knowing that the secretory nerve impulses reach the adrenal medulla through cholinergic preganglionic fibres (FELDBERG, MINZ and TSUDZIMURA, 1934; HOLLINSHEAD, 1936; YOUNG, 1939; HILLARP, 1946; STÖHR, 1957). Recent histochemical work has shown that the distributions of acetylcholinesterase (AChE) and non-specific cholinesterase (nsChE) in the adrenal medulla of the rat somewhat differ from each other (ERÄNKÖ, 1958, 1959). The present work demonstrates that denervation causes a loss of AChE activity, but does not much affect the activity of nsChE in the rat adrenal.

METHODS

Experimental procedure. Adult healthy male albino rats were unilaterally denervated by dividing the left splanchnic nerve under the diaphragm. In the first experiments, the tissues above the left adrenal were also removed to make certain that the denervation was complete. Since this operation occasionally resulted in the appearance of necrotic areas in the adrenal, probably due to interference with the blood supply of the adrenal, it was later abandoned, and denervation was carried out by dividing the splanchnic nerve only. The changes observed with the present histochemical techniques were identical in both cases.

The number of denervated rats was 34. Four of them were killed 5–9 days, 17 rats 1–2·5 months and 13 rats 3–6 months after the denervation. Both adrenals were removed and sectioned with a freezing microtome at 20 μ, fresh or after fixation in calcium formol. The sections of the left, denervated, and right, intact, adrenals were floated on slides in pairs to guarantee identical treatment, the right adrenals serving as controls. These control adrenals were found to be histochemically identical, as far as AChE and nsChE are concerned, with adrenals from entirely untreated rats.

Histochemical techniques. Cholinesterases were demonstrated with KOELLE's (1951) improved method, using acetyl- and butyrylthiocholine as substrates. α-Naphtyl acetate was also used as a substrate, following the method given by PEARSE (1953), with the exception that a higher concentration of the coupling agent, diazotate of 5-nitroanisidine (Brentamine Fast Red B Salt, I.C I. Ltd.) was used (ALLEN, ERÄNKÖ and HUNTER, 1958).

Instead of di-*iso*propylfluorophosphate, as recommended by KOELLE (1951), 62.C.47 (1:5-*bis*-(4-trimethylammonium-phenyl)pentan-3-one di-iodide (BURGEN, 1949) and tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) (ALDRIDGE, 1953) were used as inhibitors (PEPLER and PEARSE, 1957; ERÄNKÖ, 1958, 1959). In a concentration of 10^{-6} M, *iso*-OMPA completely abolished the reaction obtained with butyrylthiocholine and the eserine-sensitive component of the reaction with α-naphtyl acetate.

The reaction obtained with butyrylthiocholine and 10^{-5} M-62.C.47 was interpreted as due to nsChE activity. Essentially similar medullary distribution was also obtained with alpha-naphtyl

acetate and 10^{-5} M-62.C.47. The reaction obtained with acetylthiocholine in the presence of 10^{-6} M-*iso*-OMPA was ascribed to AChE. Incubation with both thiocholine substrates in the presence of 10^{-6} M-*iso*-OMPA and 10^{-5} M-62.C.47 resulted always in unstained slides. Incubation was carried out at 37°C for 1–1·5 hr. Before incubation in the substrate mixtures, the sections were pre-incubated with the same inhibitor(s) as used in the substrate mixture. For details see ERÄNKÖ (1959).

RESULTS

The results are summarized in Table 1, and illustrated by Figs. 1–4. Similar changes were observed in all animals regardless of the length of the period from the denervation. The AChE activity was radically affected by denervation. Almost all the medulla was entirely negative, the only reacting elements being some ganglion cells and the fibres originating from them, in which the reaction was strongly positive. Some fine cortical fibres reacted also after denervation.

TABLE 1.—EFFECT OF DENERVATION ON CHOLINESTERASES OF THE ADRENAL MEDULLA

Structure	Acetylcholinesterase		Non-specific cholinesterase	
	Control	Denervated	Control	Denervated
Capsule	−	−	+++	+++
Glomerulosa fibres	−	−	++	++
Cortical fibres	++	++	++	++
Nerve trunks	+++	−	+++	+++
Ganglion cells	+++	+++	+	+
—their fibres	++	++	+++	+++
—satellite cells	++	−	+++	++
Fine med. fibres	+++	−	−	−
Fibres around noradrenaline cells	+++	−	+++	++
Medullary cell cytoplasm	−	−	+	+

Although most of the AChE activity had disappeared, the activity of nsChE remained essentially unchanged. The only site where a slight change was observed was the nerve net associated with the noradrenaline-cell islets, in which the activity seemed to diminish somewhat. The distribution and intensity of the reaction was unchanged in all other structures which, in the normal rat adrenal, showed nsChE activity.

Since only some mid-sections were examined from each adrenal it was not possible to obtain a reliable idea of the quantitative proportions of different medullary tissues. However, it seemed that the number of nerve trunks and coarse nerve fibres was diminished in the denervated adrenals, although the nsChE reaction was unchanged in the persisting fibres.

DISCUSSION

In the present experiments denervation caused a pronounced loss in the AChE activity of the adrenal medulla. This change was evident 5 days after denervation and

was to be expected, if it is accepted that the medulla is innervated by cholinergic preganglionic fibres. Since the change produced by division of the splanchnic nerve alone was the same as that produced by division of this nerve and removal of peri-adrenal tissues, it is evident that at least a large majority of the cholinergic fibres of the rat adrenal medulla come from the splanchnic nerve. No signs of regenerating AChE positive fibres were seen even 6 months after denervation. This is of interest in view of the observation made by HOLLINSHEAD and FINKELSTEIN (1937) that, in the cat, the regeneration process is complete in about 4 months. Their inference that, in small laboratory animals, a permanent denervation would appear impossible to achieve, does not seem to apply to the rat.

The medullary ganglion cells and the fibres originating from them, as well as some fine fibres in the cortex, showed an AChE activity which was not affected by splanchnic denervation. This suggests that these structures are independent of the nerve fibres which reach the medulla through the splanchnic nerve and which, at least in the rat, seem to be responsible for the secretory innervation of the medulla. The significance of the medullary ganglion cells and the fine cortical fibres is, in spite of much study, still obscure; but the present work lends some support to the assumption that these nervous structures, which are also cholinergic, have functions other than the regulation of secretion from the adrenal medulla (see STÖHR, 1957).

The lack of changes in the activity of nsChE after denervation suggests that this enzyme is not located in the nerve fibres themselves and that it is not directly involved in the breakdown of the transmitter substance released during secretory stimulation of the adrenal medulla. It may be that this enzyme is located in the supporting nervous elements, (cf. KOELLE, 1955), a point which it has not been possible to verify with the technique used in the present work. It is somewhat surprising that the nsChE activity, which appears to be located in a fine network of fibres around the noradrenaline cell islets, is not destroyed by denervation.

SUMMARY

After division of the splanchnic nerve, acetylcholinesterase activity disappears from the nerve trunks and fine medullary nerve net of the adrenal medulla. However, medullary ganglion cells and some fine cortical fibres retain their strong acetylcholinesterase activity after denervation. The activity and distribution of non-specific cholinesterase are practically unaffected by splanchnic denervation.

Acknowledgements.—This work has been supported by grants from Finland's Cultural Fund and from the National Institute of Arthritis and Metabolic Diseases, the U.S. Public Health Service (A-1725 Cl).

REFERENCES

ALDRIDGE W. M. (1953) *Biochem. J.* **53,** 62.
ALLEN J. M., ERÄNKÖ O. and HUNTER R. L. (1958) *Amer. J. Anat.* **102,** 93.
ANTOPOL W. and GLICK D. (1940) *J. biol. Chem.* **132,** 669.
BURGEN A. S. V. (1949) *Brit. J. Pharmacol.* **4,** 219.
COUPLAND R. E. and HOLMES R. L. (1958) *J. Physiol.* **141,** 97.
ERÄNKÖ O. (1958) *Nature, Lond.* **182,** 183.
ERÄNKÖ O. (1959) *Histochemie* **1,** 257
FELDBERG W., MINZ B. and TSUDZIMURA H. (1934) *J. Physiol.* **81,** 286.
HILLARP N. A. (1946) *Acta anat.* **2,** Suppl. 4.
HOLLINSHEAD W. H. (1936) *J. comp. Neurol.* **64,** 449.

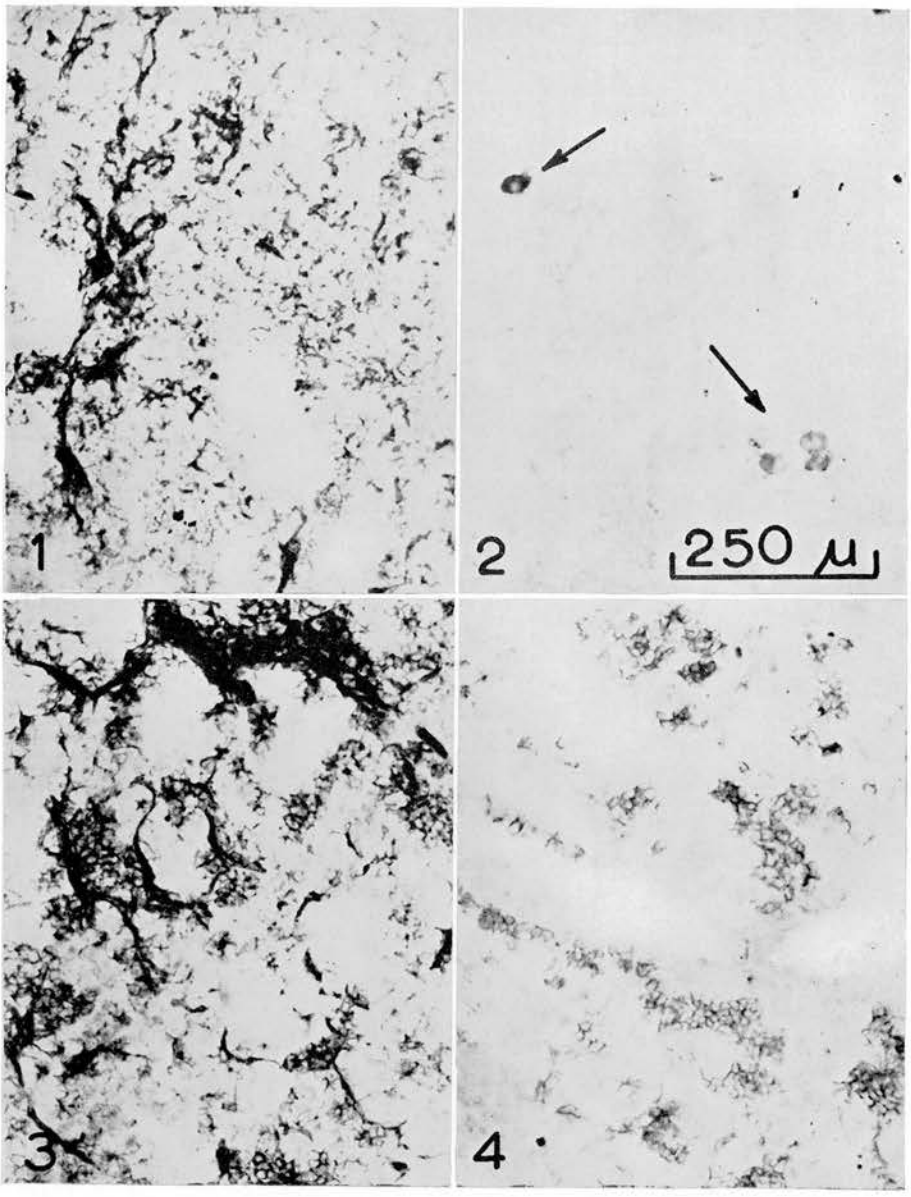


FIG. 1.—Control: AChE.

FIG. 2.—Denervated for 5 months. AChE. Arrows indicate ganglion cells.

FIG. 3.—Control: nsChE.

FIG. 4.—Denervated for 5 months. nsChE.

All figures are of the adrenal medulla of rats, ×100.

HOLLINSHEAD W. H. and FINKELSTEIN H. (1937) *J. comp. Neurol.* **67,** 215.
KOELLE G. B. (1950) *J. Pharmacol.* **100,** 158.
KOELLE G. B. (1951) *J. Pharmacol.* **103,** 153.
KOELLE G. B. (1955) *J. Pharmacol.* **114,** 167.
ORD M. G. and THOMPSON R. H. S. (1952) *Biochem. J.* **51,** 245.
PEARSE A. G. E. (1953) *Histochemistry, Theoretical and Applied.* Churchill, London.
PEPLER W. J. and PEARSE A. G. E. (1957) *J. Neurochem.* **1,** 193.
STÖHR P. Jr. (1957) Mikroskopische Anatomic des vegetativen Nervensystems *Möllendörff's Handb. d. mikr. Anat. d. Menschen.* IV/5. Springer, Berlin.
YOUNG J. Z. (1939) *J. Anat.* **73,** 540.

ERRATUM

M. LEES, J. FOLCH, G. H. SLOANE STANLEY and S. CARR (1959) *J. Neurochem.* **4,** 9.

The captions for Figs. 3 and 4 should read as follows:

FIG. 3. Hydrolysis of sulphatide *A* by saturated $Ba(OH)_2$ at 100°.

——— Release of NH_2—N as estimated by the VAN SLYKE nitrous acid method (1929).

– – – – – Liberation of galactose. Since free galactose is destroyed by alkali, these figures were calculated from the amount of galactose which remained unhydrolysed.

FIG. 4. Release of inorganic sulphur from sulphatide *A* in the presence of glacial acetic acid at 100°.

EFFECT OF PROLONGED ADMINISTRATION OF NICOTINE ON THE MEDULLARY VOLUME AND THE DISTRIBUTION OF NORADRENALINE IN THE ADRENALS OF THE RAT, THE MOUSE AND THE GUINEA PIG[1]

OLAVI ERÄNKÖ, VÄINÖ HOPSU AND LIISA RÄISÄNEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

ABSTRACT

Rats, mice and guinea pigs were daily injected with sublethal doses of nicotine for 6.5–9 months. Formalin-induced fluorescence and iodate reaction were used to demonstrate the noradrenaline-containing adrenomedullary cells, chromaffin reaction to demonstrate both catechol amines. The medullary volume and the volume of the noradrenaline-containing tissue were determined planimetrically.

In the rat, treatment with nicotine did not affect the adrenal weight but caused an 110% increase of the medullary volume. This increase was mainly due to a 470% increase in the volume of the noradrenaline-containing adrenomedullary parenchyma. No changes in the adrenal weight, the medullary volume or the distributions of the histochemical reactions were detected in the adrenals of the guinea pig and the mouse.

IT HAS been shown that prolonged administration of nicotine causes a hyperplasia of the adrenal medulla of the rat (1) and that the hyperplasia is due to an increase in the volume of the noradrenaline-containing medullary parenchyma (2).

Since these observations were made, a new histochemical method for noradrenaline has been published (3, 4). It seemed to be worth while to reinvestigate the subject using this histochemical reaction, which renders it convenient to estimate quantitatively the volume of the noradrenaline-containing medullary tissue. To study the species differences in the response to nicotine, adrenals of mice and guinea pigs were examined in addition to rats.

MATERIALS AND METHODS

Experimental

The animals were given daily subcutaneous injections of nicotine (Nicotin puriss., Dr. Theodor Schuchardt, G.m.b.H., Görlitz, Germany) in 0.2–0.4% aqueous solution, Sundays excepted.

Received February 27, 1959.

[1] Work supported by grants from Finland's Cultural Fund and from the National Institute of Arthritis and Metabolic Diseases, the U. S. Public Health Service (A-1725HH.

Rats were injected for about 9 months, guinea pigs for 8.5 months, and mice for 6.5–9 months. The dose was 0.4 mg. per rat for the first 2 months, then 0.5 mg. per rat. The mean weight of the rats at the onset of the experiment was *c.* 70 gm. and at the end *c.* 200 gm. The mice were given 0.1 mg. per animal and the mean weight of the mice at the end of the experiment was *c.* 30 gm. The guinea pigs were given 1 mg. per animal for the first 2 months, then 2 mg. per animal for 4 months, and 4 mg. per animal for 2.5 months; their mean initial weight was *c.* 240 gm. and the final weight *c.* 720 gm. The surviving animals were killed 2–4 days after the last injection by decapitation.

The iodate reaction

The left adrenals were treated for 24 hours in a saturated solution of potassium iodate for the iodate reaction (4). They were then fixed in 3.5% formaldehyde for another 24 hours and sectioned at 50 μ with a freezing microtome. The total medullary area and the area covered by iodate-positive medullary cells were planimetrically measured from 5 mid-sections of each gland, and an estimate of the relative volume of the noradrenaline-containing medullary tissue was obtained by pooling these values.

The chromaffin reaction

The right adrenals were fixed in a dichromate-formalin mixture and the medullary volume was measured from a complete series of sections, as described earlier (5).

The fluorescence method

Adrenals of other animals were fixed in formol-calcium for the demonstration of the formalin-induced fluorescence (6).

Statistical analysis

To compare the significance of the differences of the means, "Student's" *t*-test was used.

RESULTS

Behaviour of the animals

Marked differences were observed in the immediate responses of the animals to nicotine. The rats regularly showed clonic convulsions *c.* 30 sec. after each injection. These lasted about 1 min., whereafter the animals lay immovable and panted heavily. After a couple of minutes they behaved normally again.

The mice and the guinea pigs did not usually exhibit any convulsions. About 30 sec. after the injection they lay flat and panted, and the guinea pigs salivated heavily. After some minutes, normal behaviour was resumed.

Effect on adrenals

Table 1 lists the adrenal weights, the medullary volumes and the percentages of iodate-positive, *i.e.* noradrenaline-containing, cells in the medulla of the nicotine-injected and the control animals. It will be seen that the rats reacted to the treatment with nicotine by showing a 110% increase in the volume of the medulla, although no significant change in the adrenal weight occurred. By multiplying the medullary volumes by the corresponding percentages of iodate-positive tissue, it was found that the volume of

TABLE 1. EFFECT OF NICOTINE ON THE ADRENALS

Species and treatment	Adrenal weight (mg.)			Medullary volume (cu. mm.)			Iodate-positive tissue (%)		
	No.[1]	Mean	SD	No.[1]	Mean	SD	No.[1]	Mean	SD
Rats									
Nicotine-injected	5	18.7	1.5	5	2.92[2]	0.79	5	31.3[3]	9.7
Controls	5	17.6	3.8	5	1.38	0.40	5	11.8	7.5
Mice									
Nicotine-injected	7	2.2	0.5	12	0.66	0.24	14	16.3	6.6
Controls	5	2.0	0.4	9	0.52	0.10	11	17.6	3.7
Guinea pigs									
Nicotine-injected	7	313	40	7	7.96	1.69	7	0	0
Controls	4	301	63	4	8.02	2.07	4	0	0

[1] The number of glands investigated.
[2] Difference from the mean control value is significant (P~0.005).
[3] Difference from the mean control value is significant (P~0.007).

the noradrenaline-containing medullary tissue in the rat adrenals increased with 470%.

There was also a mean increase in the volume of the iodate-negative medullary tissue, the magnitude of which is of the same order in terms of cu. mm. as the mean increase in the volume of the iodate-positive tissue. However, since the original volume of the iodate-negative tissue is large, the increase is only about 60%. Moreover, this change is not statistically significant and may well be due to a sampling error. Figures 1 and 2 demon-

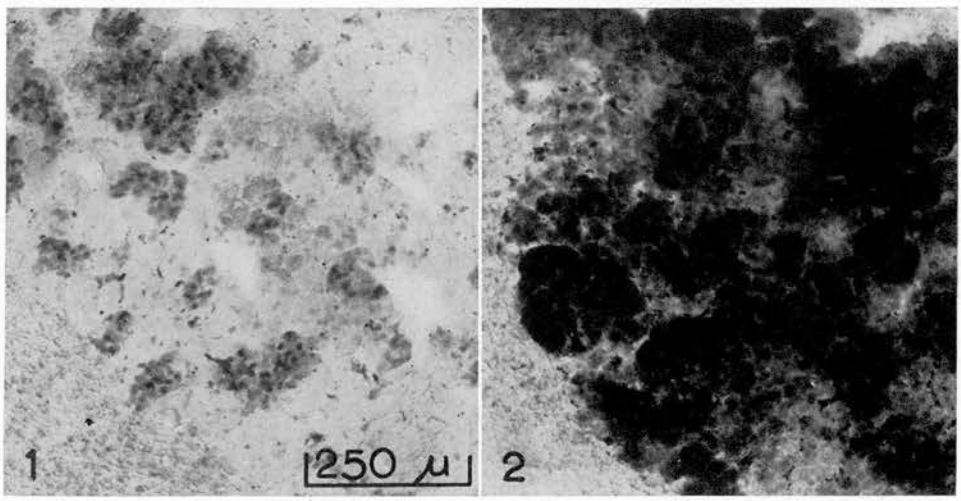


FIG. 1. Adrenal medulla of a control rat. Cortex in lower left corner. Iodate reaction, photographed at 4050 Å in transmitted light. The iodate-positive cell islets in the medulla are clearly visible, the red blood cells black. Magnification *c.* 90.

FIG. 2. Adrenal medulla of a rat injected with nicotine for 9 months. Cortex in lower left corner. A definite increase in the size of the iodate-positive islets can be seen, and the intensity of the reaction is stronger. Technique and magnification as in Fig. 1.

strate that the individual iodate-positive islets increased in size, eventually fusing together, and that the iodate reaction is more intense in the medullary cell islets of the nicotine-injected rats than in those of the controls.

Distribution of the formalin-induced fluorescence was similar to that of the iodate reaction both in the nicotine-treated and in the control rats. However, in contrast to the intensity of this reaction, the fluorescence was weaker in the medullary cells of the nicotine-treated rats than in those of the controls.

The mice and the guinea pigs showed no changes in the adrenal after treatment with nicotine. Differences in the means between the respective experimental and control groups are statistically non-significant and so small that not even a tendency towards changed values in the nicotine groups can be revealed. Also the intensities of the fluorescence and the iodate reaction remained unchanged.

DISCUSSION

The results obtained confirm the earlier observed nicotine-induced hyperplasia in the rat adrenal medulla and show that even under these conditions the two histochemical reactions used for the demonstration of the noradrenaline-containing adrenomedullary cells, *i.e.*, the formalin-induced fluorescence (2, 6) and the iodate reaction (3, 4) run parallel, the fluorescent and iodate-positive tissue being responsible for the hyperplasia. This is what can be expected, since these two reactions have been always found to be distributed in the same way under several experimental conditions (7).

It is of interest that the formalin-induced fluorescence was weaker, the iodate reaction stronger, in the medullary cell islets of the nicotine-treated rats, than in the islets of the controls, although the distributions of these two reactions were identical. Such a discrepancy has been observed earlier (7) and suggests that the two reactions may depend on different properties of the noradrenaline-containing cells, although they both selectively demonstrate them.

Neither the guinea pig nor the mouse adrenals showed any changes. This is somewhat surprising, particularly as far as the mouse is concerned, since the mouse adrenal contains—like the rat adrenal—two types of medullary cells, which the guinea pig adrenal does not, and since the dosage of nicotine given to the mice was higher in terms of body weight than that given to the other two species. In this connection it may be of interest to mention that the nodular hyperplasia induced by nicotine in the rat adrenal medulla is accompanied by pronounced changes in the distributions of cholinesterases and acid phosphatase (8), while no such changes were detected in the adrenals of similarly treated mice or guinea pigs.

Whatever the reason for the difference in the adrenal responses to nico-

tine between the species examined, the present work demonstrates once again that generalizations from one species to another may lead to entirely misleading results.

REFERENCES

1. STAEMMLER, M.: *Virchow's Arch.* **295**: 366. 1935.
2. ERÄNKÖ, O.: *Acta pathol. microbiol. Scand.* **36**: 210. 1955.
3. HILLARP, N.-Å. AND B. HÖKFELT: *Acta physiol. Scand.* **36**: 210. 1953.
4. HILLARP, N.-Å. AND B. HÖKFELT: *J. Histochem. Cytochem.* **3**: 1. 1955.
5. ERÄNKÖ, O.: *Ann. Med. Exper. Biol. Fenniae* **32**: 377. 1954.
6. ERÄNKÖ, O.: *Acta anat.* **16**: Supp. 17. 1952.
7. ERÄNKÖ, O.: *Studies on the Adrenal Medulla.* Ph.D. Thesis. Edinburgh. 1958.
8. ERÄNKÖ, O., V. HOPSU AND L. RÄISÄNEN: *J. Neurochem.* In press.

Reprinted from ENDOCRINOLOGY
Vol. 65, No. 2, August, 1959
pp. 293-297

Journal of Neurochemistry, 1959. Vol. 4, pp. 332 to 337. Pergamon Press Ltd. Printed in Northern Ireland

CHANGES INDUCED BY PROLONGED ADMINISTRATION OF NICOTINE IN THE DISTRIBUTIONS OF CHOLINESTERASES AND ACID PHOSPHATASE IN THE ADRENAL MEDULLA OF THE RAT

OLAVI ERÄNKÖ, VÄINÖ HOPSU and LIISA RÄISÄNEN
Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

(*Received* 24 *February* 1959)

PROLONGED administration of nicotine causes a pronounced hyperplasia of the adrenal medulla in the rat, as was demonstrated by STAEMMLER (1935) over 20 years ago. ERÄNKÖ (1955*a*) observed that this hyperplasia is associated with about a five-fold increase in the noradrenaline content of the adrenal without any significant change in the adrenaline content. Moreover, the volume of the noradrenaline-containing medullary parenchyma, as demonstrated histochemically by formalin-induced fluorescence (ERÄNKÖ, 1951, 1952, 1955*a*,*b*), increased in proportion to the noradrenaline content, while there was no significant change in the volume of the adrenaline-containing medullary tissue.

Earlier work has indicated that the activities of enzymes such as acid phosphatase (APh) (ERÄNKÖ, 1951, 1952) and cholinesterases (ALLEN, ERÄNKÖ and HUNTER, 1958; ERÄNKÖ, 1958, 1959) are different in the regions of the two types of adrenomedullary cells of the rat. It seemed therefore to be of interest to investigate histochemically these enzymes in the hyperplastic adrenal medulla of nicotine-injected rats. The present study shows that nicotine treatment brings about marked changes in the distributions and activities of these enzymes.

MATERIALS AND METHODS

Experimental. Young male albino rats with a mean weight of about 70 gm were daily injected subcutaneously with nicotine, Sundays excepted. Nicotine (Nicotin puriss., Dr. Theodor Schuchart G.m.b.H., Görlitz, Germany) was diluted with distilled water to a concentration of 2 mg/ml. The number of injected rats was initially 60, and the daily dose was 1 mg/rat. Because of high mortality, the daily dose was reduced after 9 days to 0·4 mg/rat. This dose was maintained for about 2 months. Thereafter 0·5 mg was given daily until the end of the treatment, which lasted about 9 months. Only 16 rats survived, and their mean weight was about 200 gm at the end of the treatment.

Control rats, reared under conditions identical with those of the experimental animals but left uninjected, did not show a weight gain significantly different from that of the nicotine-injected rats.

The injected rats were killed by decapitation 1–4 days after the last injection. The variable length of the period between the last injection and killing did not affect the results. Each time any of the injected animals were killed, a number of controls were also decapitated.

Histochemical methods. Adrenals were fixed for 2–4 hr in a mixture containing 1 vol. of neutra analytical 35% (w/w) formaldehyde solution, 6 vol. of 2% (w/v) calcium chloride and 3 vol. of distilled water. They were sectioned with a freezing microtome at 20–50 μ, rinsed in distilled water, floated on clean slides, and allowed to dry on them.

Unstained dry sections were examined in the fluorescence microscope to register the fluorescent medullary cell islets, which are presumably composed of the noradrenaline-containing secretory cells (ERÄNKÖ, 1955*b*). After fluorescence photomicrography the same sections could be used for the

demonstration of cholinesterases of APh, whose distribution can be registered by ordinary photomicrography.

Cholinesterases were demonstrated using the improved method described by KOELLE (1951). However, to differentiate between acetylcholinesterase (AChE) and non-specific cholinesterase (nsChE), selective inhibitors were used, i.e. 1:5-*bis*-(4-trimethylammonium-phenyl)pentan-3-one di-iodide (62.C.47) (BURGEN, 1949), which is a specific inhibitor for acetylcholinesterase, and tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) (ALDRIDGE, 1953), which is a selective inhibitor of non-specific cholinesterase (see PEPLER and PEARSE, 1957; ERÄNKÖ, 1958, 1959). A reaction obtained with acetylthiocholine (AThCh) as a substrate in the presence of 10^{-6} M-*iso*-OMPA was ascribed to AChE, and that obtained with butyrylthiocholine (BThCh) and 10^{-5} M-62.C.47 to non-specific cholinesterase. Sections incubated with both substrates and with both of the above-mentioned inhibitors or 10^{-5} M-eserine served as controls. Before incubation in the substrate mixtures the sections were in all instances pre-incubated in a similar but substrate-free mixture containing either or both inhibitors. Details of the technique and results obtained with normal rat adrenals are described elsewhere (ERÄNKÖ, 1958, 1959).

For the demonstration of APh a modification of GOMORI's (1941) glycerophosphate—lead nitrate method was used (ERÄNKÖ, 1952).

RESULTS

Fluorescent cell islets

In agreement with the results obtained earlier (ERÄNKÖ, 1955*a*) it was found that the medullary volume and the relative amount of fluorescent medullary tissue were greatly increased in the nicotine-injected rats (Figs. 1, 2, 7, 8, 15 and 16). The increase was due to enlargement and fusion of the individual fluorescent islets rather than to increase in their number. However, the hyperplastic medullary tissue had less tendency to form rounded nodules bulging into the cortex than in the earlier work (ERÄNKÖ, 1955*a*). Presumably this difference is due to the smaller dosage of nicotine in the present study.

Cholinesterases

The histochemical reaction observed in sections of the adrenals from control and nicotine-treated rats was always absent when both types of cholinesterase were inhibited either by eserine or by a combination of 62.C.47 and *iso*-OMPA (Figs. 13 and 14). This proves that only cholinesterases are involved in the histochemical reaction.

Acetylcholinesterase. In the adrenals of the control rats, a strong AChE reaction was found in the coarse nerve trunks, in the ganglion cells, and in the fine fibres which formed a fine network over the whole medulla and which showed small ovoid, strongly positive bodies along their lengths (Fig. 3). The regions of the medullary areas corresponding to the fluorescent cell islets exhibited a stronger reaction, presumably because of the denser network of fibres around the noradrenaline-containing cells (Figs. 1, 3 and 5).

In the adrenals of the nicotine-injected rats the distribution of AChE was in many respects similar. Thus, coarse and fine nerve fibres and the ovoid bodies along the latter fibres were strongly positive. However, there were many regions in the medulla, particularly near the corticomedullary junction, where the medullary parenchyma was entirely negative (Figs. 4 and 6). These negative areas were found to correspond to the regions of the fluorescent islets, and they probably represent newly formed medullary tissue which, although the cells are capable of forming noradrenaline, is devoid of fibres with AChE activity (Figs. 2 and 4). Whether or not nerve fibres

EXPLANATIONS OF THE FIGURE PLATES

The figures have been arranged in pairs so that photomicrographs of the adrenals of the controls are on the left, those of the nicotine-treated rats on the right.

There are 3 such pairs of fluorescence photomicrographs, i.e., Figs. 1 and 2, 7 and 8, 15 and 16. In these figures the blood vessels are black, the main medullary parenchyma dark grey, the cortical tissue, present in the upper parts of Figs. 1, 2, 7, 8 and 15, light grey, and the fluorescent medullary islets white.

Below each fluorescence photomicrograph is another photomicrograph taken in transmitted light of the same section after histochemical demonstration of AChE, nsChE or APh. In these figures the blood vessels, which serve as useful land-marks when comparing the two photomicrographs of the same section, are white, regions devoid of enzyme activity grey, enzymically active regions dark grey or black. Owing to light refraction, cortical tissue, though essentially devoid of demonstrable enzyme activity, appears darker than the enzymically inactive regions of the medulla.

To facilitate the comparison of the corresponding regions in the fluorescence photomicrographs and those taken after histochemical demonstration of the enzymes, one region of fluorescent cells is indicated in each figure by two arrow-heads.

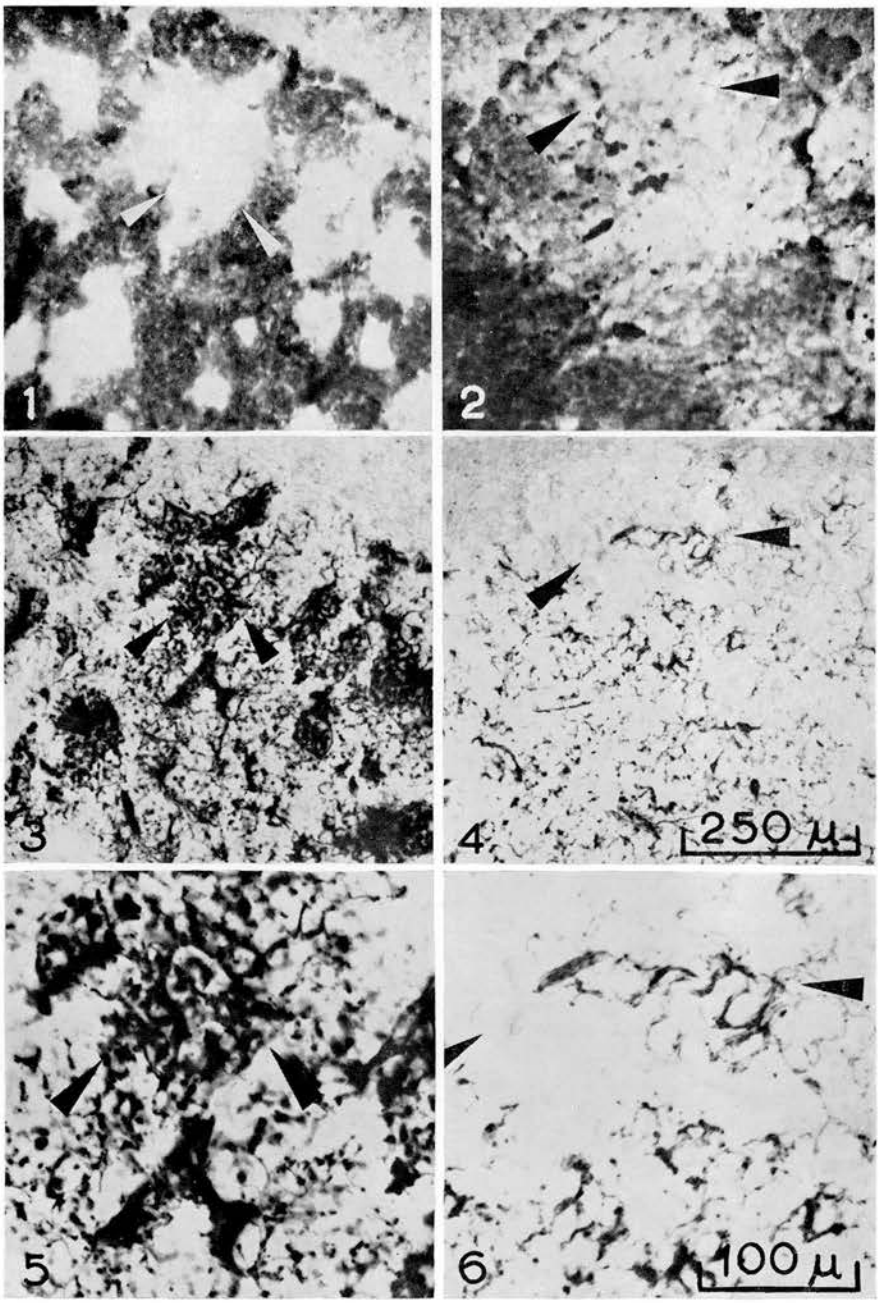


PLATE 1

FIG. 1. Fluorescence, control rat. ×90.
FIG. 2. Fluorescence, nicotine rat. ×90.
FIG. 3. Same field as in Fig. 1. AChE. ×90.
FIG. 4. Same field as in Fig. 2. AChE. ×90.
FIG. 5. Detail of Fig. 3. ×200.
FIG. 6. Detail of Fig. 4. ×200.

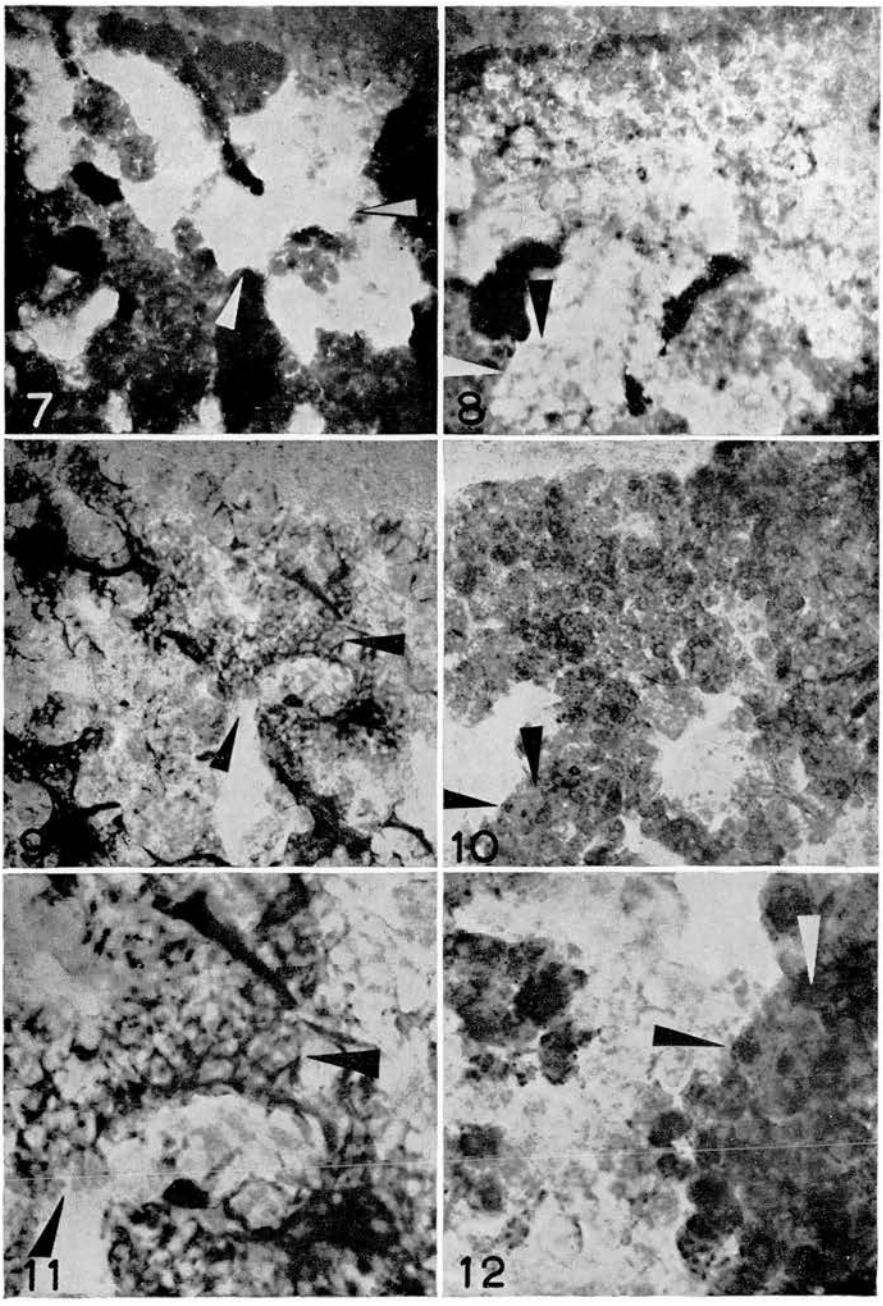


PLATE 2

FIG. 7. Fluorescence, control rat. ×90.
FIG. 8. Fluorescence, nicotine rat. ×90.
FIG. 9. Same field as in Fig. 7. nsChE. ×90.
FIG. 10. Same field as in Fig. 8. nsChE. ×90.
FIG. 11. Detail of Fig. 9. ×200.
FIG. 12. Detail of Fig. 10. ×200.

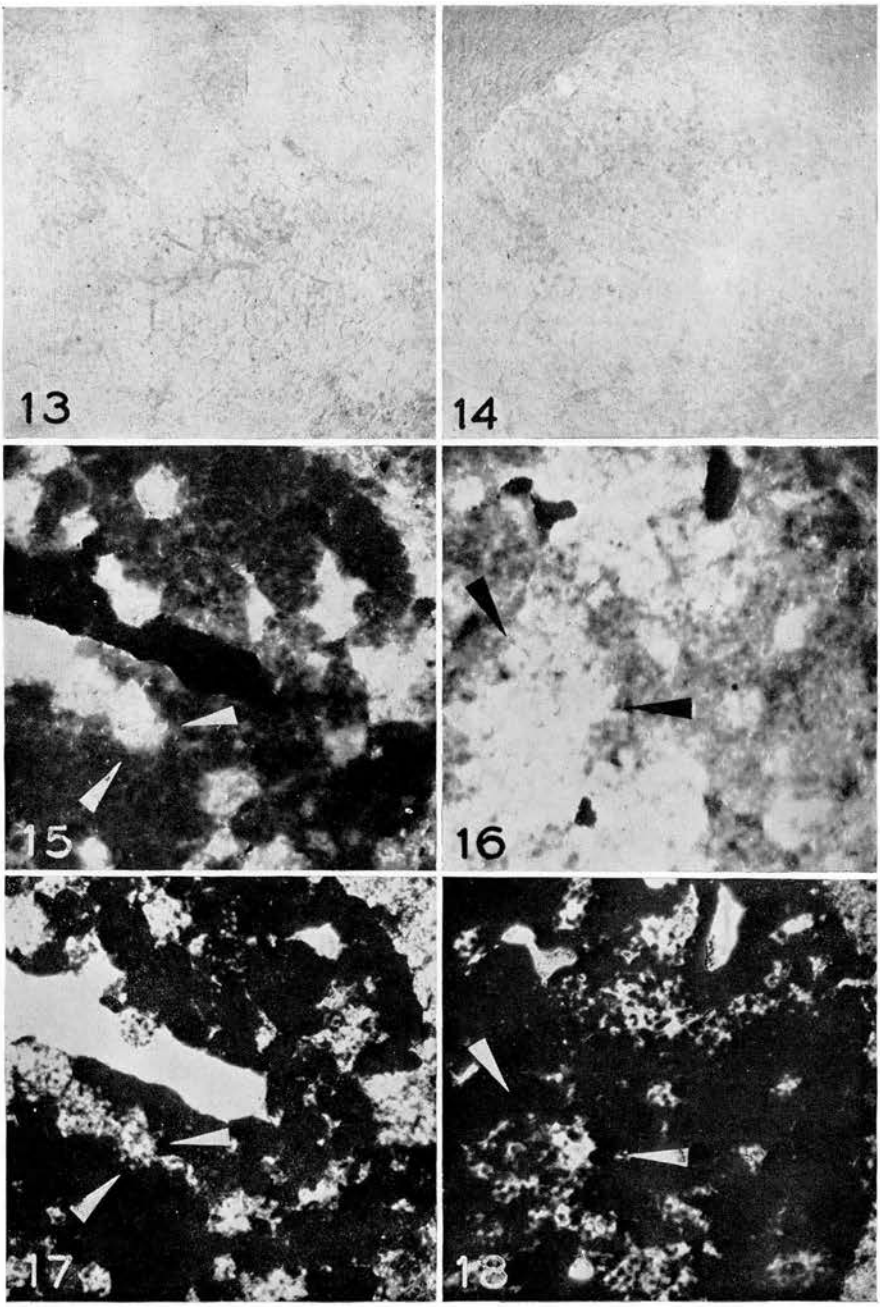


PLATE 3

FIG. 13. AThCh + BThCh + 62.C.37 + *iso*-OMPA. Control rat. ×90.
FIG. 14. AThCh + BThCh + 62.C.47 + *iso*-OMPA. Nicotine rat. ×90.
FIG. 15. Fluorescence, control rat. ×90.
FIG. 16. Fluorescence, nicotine rat. ×90.
FIG. 17. Same field as in Fig. 15. APh. ×90.
FIG. 18. Same field as in Fig. 16. APh. ×90.

were really lacking from these regions, cannot be finally decided with the methods used in the present study. In any case, such a negative reaction in the region of fluorescent medullary tissue is the more remarkable, since the positive network in similar regions of normal adrenals is even somewhat denser than in the regions of the non-fluorescent medullary cells.

Non-specific cholinesterase. In the normal rats the medullary reaction was positive in the coarse nerve fibres and the small medullary ganglia, as well as in the dense network of fibres entangling the fluorescent parenchymal cell islets (Figs. 7, 9 and 11). The remaining medullary parenchyma, which is composed of the non-fluorescent, presumably adrenaline-containing cells, and of the fine nerve fibres around these cells, reacted but weakly.

The distribution pattern of nsChE was different in the adrenal medulla of the nicotine-treated animals. There were positive coarse fibres and nerve trunks both in the regions of the cortex and the medulla but in the medulla they were overshadowed by large groups of cells with a strongly positive reaction (Fig. 10). Comparison of fluorescence photomicrographs (Fig. 8) with those taken of the same area after demonstration of nsChE (Fig. 10) showed that the fluorescent cell islets regularly exhibited a strong enzyme activity. At higher magnification it was seen that the enzyme activity, which in normal adrenals was fairly sharply restricted to the fibre network surrounding the noradrenaline-containing cells (Fig. 11), was uniformly positive in the cytoplasm of the medullary parenchymal cells of the nicotine-injected rats. In addition, a strongly positive reaction was seen in small spherical bodies between the cells, while no fibre network at all was visible (Fig. 12).

Acid phosphatase

As in earlier studies (ERÄNKÖ, 1952), APh reaction was selectively detected in the non-fluorescent areas of the medulla of the control rats, the fluorescent cell islets remaining essentially negative (Figs. 15 and 17). On the other hand, the region of the larger islets formed by the fluorescent medullary cells in the adrenals of the nicotine-injected rats gave a positive APh reaction (Figs. 16 and 18). Closer examination showed that the centre of these islets, which tended to fluoresce brighter than the periphery, showed as a rule a weaker APh reaction than the rest of the medulla (Fig. 18).

DISCUSSION

The present study confirms the earlier observation that nicotine causes an increase in the volume of the parenchyma formed by those medullary cells which fluoresce after treatment with calcium-formol, i.e. the noradrenaline-containing ones, (ERÄNKÖ, 1955*a*). It also shows that alterations in the activities of the three enzymes now investigated are restricted to the regions of the fluorescent cells.

It is of interest to note that opposite effects were caused by treatment with nicotine in the APh activity and in the AChE activity. While the former increased, the latter decreased. These changes did not occur throughout the fluorescent parenchyma; they were restricted to only parts of it, mainly to the peripheral areas of the large fluorescent islets, where the formation of new noradrenaline-containing cells had most likely occurred. The centre of these islets, probably representing 'old' fluorescent cells, showed no, or less, changes in the APh and AChE activities.

One might speculate that the absence of AChE activity from these regions is simply due to the formation of new parenchymal tissue without corresponding formation of nerve fibres. Similarly, the strong APh activity could be taken as a sign of the primitive nature of the newly formed cells. However, it was shown that a single injection of 0·25 mg of reserpine causes a complete disappearance of noradrenaline in 24 hr, as demonstrated histochemically with the iodate reaction (HILLARP and HÖKFELT, 1955), from the noradrenaline-containing hyperplastic medullary cell islets of nicotine-treated rats (unpublished). Since similar reserpine-induced depletion is abolished in normal rats by splanchnic denervation (ERÄNKÖ and HOPSU, 1958), it would seem that the hyperplastic noradrenaline cell islets in the adrenals of the nicotine-injected rats are capable of responding to nervous stimuli by increased secretion of noradrenaline.

In contrast to the changes in the activities of APh and AChE, which were limited to a part of the fluorescent cell islets, nicotine treatment caused the appearance of nsChE throughout the fluorescent islets, which in the adrenals of the control rats exhibited little activity of nsChE. Thus, not only the newly formed noradrenaline-producing cells were affected, but also those noradrenaline-containing cells which were present before nicotine administration. The significance of the changes in nsChE are at present obscure, but it is of interest to note that denervation of normal adrenals is not accompanied by a loss of nsChE from the networks around the fluorescent cells, in spite of a complete disappearance of histochemically demonstrable AChE from the medullary nerve net (ERÄNKÖ, 1958; ERÄNKÖ, HOPSU and RÄISÄNEN, 1959).

SUMMARY

Hyperplasia of the medulla was induced in male albino rats by daily subcutaneous injections of nicotine for 9 months. The newly formed medullary tissue which exhibited a strong fluorescence in ultraviolet light after fixation in calcium-formol differed histochemically from the fluorescent medullary tissue in normal rat adrenals. Fibres exhibiting a positive reaction for acetylcholinesterase were fewer or lacking in the newly formed medullary parenchyma of nicotine-treated rats. The adrenals of these animals, on the other hand, showed a strong activity of non-specific cholinesterase in the cytoplasm of all fluorescent medullary cells and a strongly positive acid phosphatase reaction in the peripheral parts of the fluorescent islets which in normal adrenals give essentially negative reactions for both enzymes.

Acknowledgements—This work has been supported by grants from Finland's Cultural Fund and the National Institute of Arthritis and Metabolic Diseases, the U.S. Public Health Service (A-1725).

REFERENCES

ALDRIDGE W. N. (1953) *Biochem. J.* **53,** 62.
ALLEN J. M., ERÄNKÖ O. and HUNTER R. L. (1958) *Amer. J. Anat.* **102,** 93.
BURGEN A. S. V. (1949) *Brit. J. Pharmacol.* **4,** 219.
ERÄNKÖ O. (1951) *Acta physiol. scand.* **25,** Supp. 89, 22.
ERÄNKÖ O. (1952) *Acta anat.* **16,** Supp. 17.
ERÄNKÖ O. (1955*a*) *Acta path. microbiol. scand.* **36,** 210.
ERÄNKÖ O. (1955*b*) *Nature, Lond.* **175,** 88.
ERÄNKÖ O. (1958) *Nature, Lond.* **182,** 183.
ERÄNKÖ O. (1959) *Histochemie* **1,** 257.
ERÄNKÖ O. and HOPSU V. (1958) *Endocrinology* **62,** 15.

ERÄNKÖ O., HOPSU V. and RÄISÄNEN L. (1959) *J. Neurochem.* **4,** 264.
GOMORI G. (1941) *Arch. Path.* **32,** 189.
HILLARP N. Å. and HÖKFELT B. (1955) *J. Histochem. Cytochem.* **3,** 1.
KOELLE G. B. (1951) *J. Pharmacol.* **103,** 153.
PEPLER W. J. and PEARSE A. G. E. (1957) *J. Neurochem.* **1,** 193.
STAEMMLER M. (1935) *Virchows Arch.* **295,** 366.

Reprinted from THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 9, No. 1 January, 1961

CYTOPHOTOMETRIC STUDY ON THE CHROMAFFIN REACTION, THE IODATE REACTION AND FORMALIN-INDUCED FLUORESCENCE AS INDICATORS OF ADRENALINE AND NORADRENALINE CONCENTRATIONS IN THE ADRENAL MEDULLA*· †

OLAVI ERÄNKÖ AND LIISA RÄISÄNEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received for publication January 25, 1960

The chromaffin reaction (2, 10, 13) is the classic method for the demonstration of catecholamines; it colors brown both the adrenaline- and the noradrenaline-containing cells in the adrenal medulla. The latter cells can be selectively demonstrated with the aid of the fluorescence induced in them by formalin fixation (3, 5) or by treating them with a saturated solution of potassium iodate (11, 12), which produces a brown precipitate with noradrenaline; adrenaline does not give these reactions.

These three methods have been shown not only to demonstrate the presence and distribution of adrenaline and noradrenaline but also to give at least a rough idea of the concentration of these catecholamines under experimental conditions (e.g. 6, 7). However, so far as we know, the intensities of these reactions have not earlier been actually measured in sections and compared with the results of independent chemical determinations. We decided therefore to undertake such an investigation, the results of which are reported in the present paper.

METHODS

Experimental: Adult albino rats served as experimental animals, the total number of which was 111. Depletion of catecholamines was induced in 27 animals by injections of reserpine and in 30 rats by running in a treadmill; the number of untreated controls was 54. Reserpine (Serpasil, Ciba) was given in 4 consecutive days subcutaneously; the dose was 0.4 mg/100 gm body weight. Running in the treadmill lasted overnight in two consecutive days; in the day after the first running night the rats were allowed to rest but no food was given. The animals were killed in groups 0–24 days after the treatment, and about the same number of similar but untreated controls were killed each time. The treatment of the adrenals from the experimental and the control animals was identical in all respects. Details of the effects of these treatments will be reported elsewhere; here it is sufficient to state that a wide range of catecholamine concentrations in the adrenal medulla was thus obtained.

Both adrenals of each animal were quickly removed and halved. One half of the left adrenal was used for the chromaffin reaction, the other half for the iodate reaction. One half of the right adrenal served for studying the formalin-induced fluorescence, the other one for the chemical determination of adrenaline and noradrenaline.

Histochemical methods: (1) *The chromaffin reaction.* A mixture containing 1 part of 35% HCHO and 19 parts of 3.5% $K_2Cr_2O_7$ was prepared fresh each time; the adrenal halves were fixed in it overnight; thereafter they were postfixed in 3.5% HCHO for 3–5 days.

(2) *The iodate reaction.* After immersion overnight at room temperature in a solution of KIO_3 saturated at 37°C, the halves of adrenals were fixed in 3.5% HCHO for 3 days.

(3) *Formalin-induced fluorescence.* The fluorescence was brought up by fixation for 4–6 hours in a mixture containing 1 volume of 35% HCHO, 6 volumes of 2% $CaCl_2$ and 3 volumes of H_2O.

After completed fixation in the different media, sections were cut at 50 μ with a freezing microtome. The sections were rinsed in distilled water and mounted in glycerol.

Quantitative measurement of histochemical reactions: The optical densities of the chromaffin and iodate reactions were measured with the aid of a microphotometer consisting of a Leitz Ortholux microscope with the standard incandescent lamp, supplied with a 5 amp current from an electromagnetic current stabilizer. The magnified image of a small area of the section was projected on the measuring plane with the aid of a 40× ob-

* Presented at the Second Scandinavian Summer Meeting of Biochemistry, Medical Chemistry, Pharmacology and Physiology, Turku, Finland, August 29, 1959.

† This work was supported by grants from the Sigrid Jusélius Foundation and the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

jective and 10× ocular at a linear magnification of 1200×. In the image plane, a round aperture 2 mm in diameter limited the field to be measured, corresponding to a diameter of 1.7 μ in the plane of the section. Above the aperture there was a diffusing ground glass and above it an RCA 1P28 photomultiplier tube connected with a battery voltage source and a galvanometer whose sensitivity could be adjusted with a potentiometer.

A cobalt glass filter together with a $CuSO_4$ cuvette provided a suitable density range for the measurement of the iodate reaction. A green filter and $CuSO_4$ served the same purpose for the chromaffin reaction. The density was measured by setting the galvanometer at zero density (100% transmission) when an area of the slide free of tissue was under the microscope; then the medulla was moved into the field and a new density reading made.

Of each animal, two sections were measured, taking three density readings from the medullary part of each section, obtaining thus six figures for both the chromaffin and the iodate reaction. Visual selection of the areas to be measured was necessary so as to be sure that medullary tissue is involved and that the light absorption is even within the area to be measured; thus it was also possible to measure separately the optical density of the almost negative medullary background and the positive cell islets in the sections treated for the iodate reaction. The danger of subjective bias thus introduced was rendered insignificant by making the density measurements without any knowledge of the results of the corresponding chemical determinations.

Fluorescence intensity was determined by photographic photometry. Formalin-fixed sections were photomicrographed on a Perutz Peromnia 25 roll film, always exposing sections from adrenals of both experimental and control animals on the same film. The standard Leitz mercury lamp was used as an exciting light source, using a $CuSO_4$ cuvette for absorbing the infrared light and a 1 mm Jena UG 1 filter for absorbing the visible light. A yellow-green filter giving a complete extinction with the directly passing light served as a secondary filter above the 10× objective. The linear magnification in the image plane was ×74. The fluorescence intensity was determined from the photographic negatives by density measurements with a calibrated and stabilized photoelectric densitometer and by subsequent transformation of the density figures into values linear with the intensity of light falling on the photographic emulsion.

Chemical determination of catecholamines: The half of the right adrenal taken for chemical determination was immediately frozen on the tissue holder of a freezing microtome and frozen sections were cut at 50μ with a cold knife. The sections were freeze-dried and pieces of pure medulla were removed by microdissection from the dry sections under a stereomicroscope. The pieces were weighed to the nearest μg and determinations of catecholamines were carried out fluorimetrically after separation by paper chromatography, using oxidation with $K_3Fe(CN)_6$, stabilization with ascorbic acid and tautomerization into fluorescent adrenolutines with NaOH. The method was essentially that described earlier (4) but a higher concentration of ascorbic acid was used, according to Euler and Floding (9).

In a part of the material in the running experiment, the medullary pieces were extracted directly with 0.4% NaH_2PO_4, and adrenaline and noradrenaline were transformed into fluorescent compounds as above. The proportion of noradrenaline in the mixture was determined by making fluorescence measurements at two exciting wavelengths, 366 mμ and 436 mμ. Noradrenaline percentage was obtained with the aid of calibration curves prepared for each series of determinations from standard mixtures of adrenaline and noradrenaline. The method can be regarded as a modification of that published by Cohen and Goldenberg (1). The values obtained with it were comparable with those obtained after chromatographic separation.

RESULTS

The results are illustrated in Figures 1–3. Each point represents a mean of one experimental group consisting of 5–8 animals. Since the distribution of noradrenaline cell islets varies in different parts of the medulla and only two sections of one half adrenal were used for obtaining an estimate of each variable, the samples obtained from a single animal were not sufficiently representative of the whole medulla to allow comparison of the parameters on an individual base. Furthermore, since the number of photometric measurements had to be limited for practical reasons, the small number of measurements per adrenal half further increased the sampling error.

This error can be compensated for by pooling the information derived from several identically treated animals. The plane in which the adrenals were halved varied randomly from gland to gland, thus making the pooled mean of each group fairly representative. Pooling compensates also for the sampling error within sections due to relatively few points of measurement.

Changes due to noradrenaline depletion were

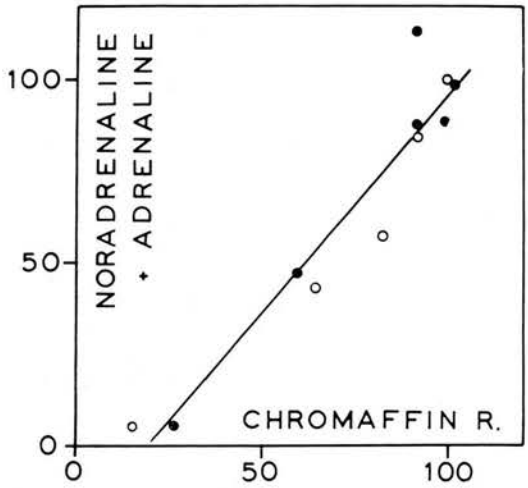


FIG. 1. Regression of the optical density of the chromaffin reaction on the catecholamine concentration. Each circle represents a mean value of a group; full circles, running experiment; open circles, reserpine experiment. The values are expressed as % of control value.

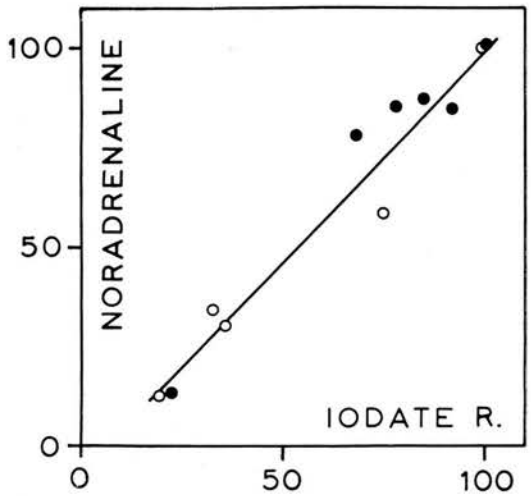


FIG. 2. Regression of the optical density of the iodate reaction on the noradrenaline concentration. Each circle represents a mean value of a group; full circles, running experiment; open circles, reserpine experiment. The values are expressed as % of control value.

TABLE I

Regression of the Optical Density of the Chromaffin Reaction (x) on the Catecholamine Concentration (y)

Variates transformed into % of control value

Source of variation	Q	f	s^2	v^2	P
Regression.....	12,504	1	12,504	78	<0.001
Residual.....	1,439	9	160	—	—
Total.....	13,943	10	—	—	—

$n = 11$, $y = 1.18x - 21.8$, $\bar{y} = 67.1$, $\bar{x} = 75.1$, correlation coefficient $r = 0.947$

TABLE II

Regression of the Optical Density of the Iodate Reaction (x) on the Noradrenaline Concentration (y)

Variates transformed into % of control value

Source of variation	Q	f	s^2	v^2	P
Regression.....	10,992	1	10,992	225	<0.001
Residual.....	439	9	49	—	—
Total.....	11,431	10	—	—	—

$n = 11$, $y = 1.05x - 6.6$, $\bar{y} = 62.1$, $\bar{x} = 65.4$, correlation coefficient $r = 0.962$

accompanied by corresponding changes in the intensities of the fluorescence and of the iodate reaction in the noradrenaline-containing medullary cell islets, while the changes in the other medullary cells were small. Therefore, noradrenaline concentrations were compared only with the microphotometric values obtained from the specific medullary cell islets, neglecting the minor changes in the rest of the medulla.

The mean density values of the chromaffin reaction and the iodate reaction, the total catecholamine concentration and the noradrenaline concentration in each group were linearly transformed by expressing both the density and the catecholamine concentration in terms of percentage of the mean control value. Since the animals belonging to the reserpine-treated group were of different age from those in the running group, and since the methods used were slightly different, the relative values were separately calculated from the respective control means of these two experiments, based on 26 controls in the reserpine and 28 controls in the running experiment.

While reproducibility from day to day allowed pooling of the densitometric data and the chemical data of all the controls in each experiment, daily variations in the fluorometric data were considerable because of variation from one photographic film to another, the intensity of the ultraviolet light exciting the fluorescence etc. Therefore, the fluorescence values were expressed as percentage of the mean value of those controls killed each time together with the experimental animals and not as percentage of the mean obtained from all the controls in each experiment.

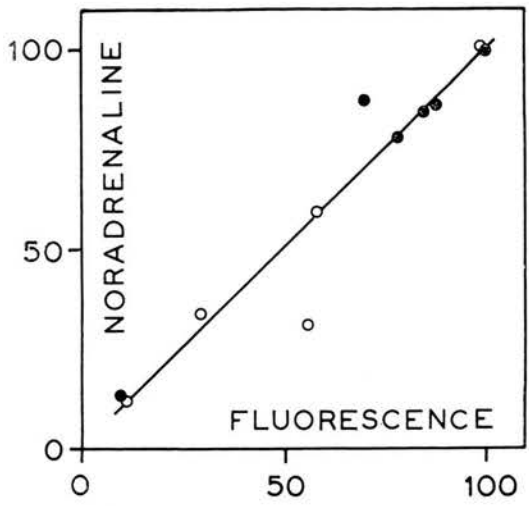


FIG. 3. Regression of the intensity of the formalin-induced fluorescence on the noradrenaline concentration. Each circle represents a mean value of a group; full circles, running experiment; open circles, reserpine experiment. The values are expressed as % of control value.

TABLE III

Regression of the Intensity of the Formalin-Induced Fluorescence (x) on the Noradrenaline Concentration (y)

Variates transformed into % of control value

Source of variation	Q	f	s^2	v^2	P
Regression.....	10,480	1	10,480	99	<0.001
Residual.....	951	9	106	—	—
Total.....	11,431	10	—	—	—

$n = 11$, $y = 0.98x + 1.3$, $\bar{y} = 62.1$, $\bar{x} = 62.2$, correlation coefficient $r = 0.917$

The transformation of the density, fluorescence and concentration values into percentage values actually means linear adjustment of the scales of the coordinates and setting the control means at 100%. If the histochemical measurements agree with the chemical determinations the points should then fall along a line from the point $x = 100\%, y = 100\%$ to the point $x = 0\%, y = 0\%$.

The regression lines were calculated according to the method of least squares; they are shown in Figs. 1–3 together with the actual data, represented by circles. The corresponding analyses of variance, regression equations and correlation coefficients are given in Tables I–III.

From Fig. 1 and Table I it is evident that the density of the chromaffin reaction does not fall to zero with a zero concentration of the catecholamines. This is due to the fact that scattering caused some non-specific loss of light under the conditions of measurement. Actually the sections from the adrenals in which the catecholamine concentration was near zero were practically colorless, *i.e.* the chromaffin reaction was negative. Similar or greater nonspecific light loss was also detected in measurements made of cortical areas, which always exhibited a negative chromaffin reaction.

The distribution of the points fairly closely around the regression line in Fig. 1 as well as the analysis of variance and the correlation coefficient given in Table I show that the density of the chromaffin reaction is closely correlated with the catecholamine concentration.

The same also applies to the correlations of the iodate reaction, on the one hand, and the fluorescence intensity, on the other, with the noradrenaline concentration (Figs. 2 and 3, Tables 2 and 3). Nonspecific light loss explains the fact that the medullary density of the sections with the iodate reaction was somewhat higher than might have been expected from the noradrenaline concentration (Fig. 2); actually hardly any color was evident in sections with a catecholamine concentration below 10% of the control value.

The regression line in Fig. 3 shows a slope of c. 45°, indicating little interference by the background fluorescence. Since the fluorescence is emitted by the section itself, no interference by light scattering is indeed to be expected.

DISCUSSION

The results presented demonstrate that the histochemical reactions employed followed with reasonable accuracy the changes in the total catecholamine or noradrenaline concentration under the experimental conditions of the present study. Since both reserpine treatment and running gave similar results in this respect, it is likely that the same would also apply to other means of altering the function of the adrenal medulla.

It is not possible to say from the data obtained to what degree the limited scatter of the points around the regression lines is due to the sampling error, error of measurement, or a failure of the histochemical reactions to faithfully indicate catecholamine concentrations. It must also be emphasized that, since each point represents a mean obtained from several values, each one obtained from a single animal, the conclusions derived from the data cannot be extended to single adrenals or single adrenal sections. However, although these reservations must be made, the results make a strong argument for the validity

of the histochemical reactions in the quantitative sense.

The good correlations between the histochemical and the chemical measurements, although they suggest a fairly close interdependence of the variables, do not guarantee by any means that the histochemical methods used depend exclusively on the presence of the catecholamines or any one of them. Earlier studies suggest indeed that under certain conditions the intensities of the iodate reaction and the formalin-induced fluorescence may change to opposite directions (8). Substances fluorescing after formalin fixation are many, a fact lowering the specificity for noradrenaline which may be attributed to the fluorescence method considerably. On the other hand, a positive iodate reaction is dependent on the mode of storage of noradrenaline in the cytoplasm (12). A negative iodate reaction may therefore be obtained even if noradrenaline is present.

For all the above reasons it is necessary to exercise sufficient care particularly in interpreting quantitatively the three histochemical reactions. In certain cases it may be sufficient to use exclusively the histochemical methods, but no doubt many pitfalls can be avoided by making use whenever possible of both chemical and histochemical methods in conjunction. When this is done, quantitative measurements of the intensities of the formalin-induced fluorescence, the chromaffin reaction and the iodate reaction can provide valuable information of catecholamine concentration in tissue constituents too small for measurements with more specific chemical methods.

SUMMARY

Variable depletion of adrenal catecholamines was caused in rats by injections of reserpine and muscular work. The intensities of the chromaffin reaction, the iodate reaction and formalin-induced fluorescence in adrenal sections were measured by cytophotometry and compared with chemically determined concentrations of adrenaline and noradrenaline in the adrenal medulla. A close correlation was observed between the optical density of the chromaffin reaction and the total catecholamine concentration, as well as between the intensity of the formalin-induced fluorescence, the optical density of the iodate reaction and the noradrenaline concentration.

REFERENCES

1. Cohen, G., and Goldenberg, M.: The simultaneous fluorimetric determination of adrenaline and noradrenaline in plasma. *J. Neurochem.* **2:** 58–70, 1957.
2. Coupland, R. E.: Observations on the chromaffin reaction. *J. Anat.* **88:** 142–151, 1954.
3. Eränkö, O.: On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. *Acta Anat.* **16:** Supp. 17, p. 60, 1952.
4. Eränkö, O.: Histological sampling, chromatographic separation, and determination of adrenaline and noradrenaline. *Ann. Med. Exp. Biol. Fenn.* **32:** 392–401, 1954.
5. Eränkö, O.: Histochemistry of noradrenaline in the adrenal medulla of rats and mice. *Endocrinology* **57:** 363–368, 1955.
6. Eränkö, O., and Hopsu, V.: Effect of reserpine on the histochemistry and content of adrenaline and noradrenaline in the adrenal medulla of the rat and the mouse. *Endocrinology* **62:** 15–23, 1958.
7. Eränkö, O., and Räisänen, L.: Adrenaline and noradrenaline in the adrenal medulla during postnatal development of the rat. *Endocrinology* **60:** 753–760, 1957.
8. Eränkö, O., Hopsu, V., and Räisänen, L.: Effect of prolonged administration of nicotine on the medullary volume and the distribution of noradrenaline in the adrenals of the rat, the mouse and the guinea pig. *Endocrinology* **65:** 293–297, 1959.
9. von Euler, U. S., and Floding, I.: A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta Physiol. Scand.* **33:** Supp. 118, 45–56, 1955.
10. Henle, J.: Ueber das Gewebe der Nebenniere und der Hypophyse. *Z. rat. Med.* **24:** 143–152, 1865.
11. Hillarp, N.-Å., and Hökfelt, B.: Evidence of adrenaline and noradrenaline in separate adrenal medullary cells. *Acta Physiol. Scand.* **30:** 55–68, 1953.
12. Hillarp, N.-Å., and Hökfelt, B.: Histochemical demonstration of noradrenaline and adrenaline in the adrenal medulla. *J. Histochem. Cytochem.* **3:** 1–5, 1955.
13. Kohn, A.: Ueber den Bau und die Entwicklung der sog. Carotisdrüse. *Arch. mikr. Anat.* **56:** 81–148, 1866.

Acta physiol. scand. 1961. 51. 239—246

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Distribution and Concentration of Adrenaline and Noradrenaline in the Adrenal Medulla of the Rat Following Reserpine-Induced Depletion

By

OLAVI ERÄNKÖ and VÄINÖ HOPSU

Received 9 November 1960

Abstract

ERÄNKÖ, O. and V. HOPSU. *Distribution and concentration of adrenaline and noradrenaline in the adrenal medulla of the rat following reserpine-induced depletion.* Acta physiol. scand. 1961. *51.* 239—246. — Adrenaline and noradrenaline concentrations in the adrenal medulla were determined after chromatographic separation by fluorometry. The chromaffin reaction, the iodate reaction and formalin-induced fluorescence were used as histochemical reactions and their intensities in the sections were quantitatively measured by microphotometry. Four mg/kg of reserpine daily for 4 days caused a pronounced loss of adrenaline and noradrenaline and recovery took about 3 weeks. During the recovery adrenaline and noradrenaline concentrations increased proportionally. The chemical and the histochemical measurements gave identical results. There was no evidence of local resynthesis of noradrenaline in excess of that of adrenaline. The results are at variance with observations reported earlier from other laboratories.

A large number of studies have been published demonstrating a pronounced loss of catecholamines from the adrenal medulla after administration of reserpine (CARLSSON and HILLARP 1956, ERÄNKÖ and HOPSU 1958, CAMANNI, LOSANA and MOLINATTI 1958, CALLINGHAM and MANN 1958 a, b, COUPLAND 1959, and others). The recovery of the medullary hormones after such depletion has been much less studied; we have found only the papers by CALLINGHAM and MANN (1958a, b) and COUPLAND (1959) to deal with it. CALLINGHAM and MANN (1958a, b) found that the noradrenaline content of the adrenals in

a few days not only returned to normal but increased greatly above it, while the adrenaline content reached the control value much more slowly; they studied only the content of catecholamines. COUPLAND (1959) used also histochemical reactions to investigate the distributions of adrenaline and noradrenaline; he reported that during the recovery phase noradrenaline is present in greater than normal amounts in those regions of the medulla which are normally concerned with storing adrenaline. Both CALLINGHAM and MANN (1958a) and COUPLAND (1959) concluded that methylation of noradrenaline is the rate-limiting factor in the synthesis of adrenaline.

In the present report observations are described in which both chemical and quantitative histochemical methods were used for studying the changes in the adrenomedullary catecholamines after reserpine administration.

Material and Methods

Experimental

Adult albino rats were used. In preliminary experiments, varying single doses of reserpine were injected and the depletion and recovery of the catecholamines was examined using only histochemical techniques (chromaffin reaction, iodate reaction, formalin-induced fluorescence). Similar experiments were then made using both histochemical and chemical methods of analysis.

The main experiments were carried out by using, first, 2 mg of reserpine per kg body weight subcutaneously daily for 3 consecutive days. This is twice the dosage used by CALLINGHAM and MANN (1958a). The daily dose was in later work increased to 4 mg/kg, and the rats were injected for 4 days. The animals were killed 1—24 days after the last injection by decapitation, together with about the same number of similar but untreated control rats. The adrenals were thereupon removed and halved. One of the 4 halves was used for chemical determinations, the remaining 3 ones for histochemical studies.

Chemical methods

The piece of adrenal was frozen on the microtome table and fresh sections were cut at 50 μ. The sections were frozen-dried and pieces of pure medulla dissected out. These pieces were weighed to the nearest μg, the catecholamines were separated by paper chromatography in a phenol-hydrochloric acid system and the separated amines were then determined fluorometrically. The fluorometric method used was essentially that used earlier in this laboratory (ERÄNKÖ 1954) but a higher concentration of ascorbic acid was used as proposed by EULER and FLODING (1955). This method is based on oxidation by ferricyanide; it was first described by EHRLÉN.

Chromaffin reaction

Adrenal halves were immersed in a mixture of 1 volume of 35 % formaldehyde and 19 volumes of 3.5 % potassium dichromate for 24 hours and, subsequently, in 3.5 % formaldehyde for another 24 hours. Frozen sections were then cut at 50 μ; these were mounted in glycerol.

Iodate reaction

Adrenal halves were immersed in a saturated solution of potassium iodate (HILLARP and HÖKFELT 1955) for 24 hours and, subsequently, in 3.5 % formaldehyde for 24 hours. Sections cut at 50 μ were mounted in glycerol.

Formalin-induced fluorescence

Adrenal halves were fixed for 3—4 hours in a solution containing 1 volume of 35 % formaldehyde, 6 volumes of 2 % calcium chloride and 3 volumes of distilled water. Sections cut at 50 μ were mounted in glycerol and studied for fluorescence.

Quantitative measurement of histochemical reactions

The optical densities of the chromaffin reaction and the iodate reaction were measured with a microphotometer. Details of the apparatus and the method have been described elsewhere (ERÄNKÖ and RÄISÄNEN 1960). Measurements were carried out in two sections of each adrenal half, taking density readings from different parts of the medulla. In sections treated for the iodate reaction, the densities of the medullary background, consisting of the almost iodate-negative adrenaline-containing cells, and of the specific islets composed of the iodate-positive noradrenaline-containing cells were recorded separately. The density measurements were made against a blank area on the slide, setting the photometer reading at zero density (100 % transmission). The results are expressed in terms of density (*i. e.*, the negative logarithm of the transmission).

The fluorescence intensity was measured using photographic photometry (for details see ERÄNKÖ and RÄISÄNEN 1960). Readings were taken from both the weakly fluorescent medullary background and the fluorescent medullary islets, which correspond to the iodate-negative and -positive parts of the medulla, respectively. The intensity of the medullary fluorescence varied for methodological reasons somewhat from day to day (see ERÄNKÖ and RÄISÄNEN 1960); therefore, the fluorescence intensity was expressed in terms of per cent of the mean intensity of the corresponding region of the adrenal medulla of those control animals killed together with the injected animals.

Results

In the preliminary experiments with a single dose of reserpine, variable loss of medullary substances giving the three histochemical reactions was observed. With lower doses only some medullary cells lost their chromaffin reaction, while the normally iodate-positive and fluorescent medullary cell groups exhibited a total loss or a marked decrease in the intensity of these two histochemical reactions. This suggests in agreement with earlier observations (ERÄNKÖ and HOPSU 1958) that the effect was limited mainly to the noradrenaline-containing cells.

With larger single doses of reserpine and three daily injections of 2 mg/kg, the chromaffin reaction was negative or of decreased intensity in larger areas of the adrenal medulla, showing that the treatment had also caused a loss of adrenaline. In all these experiments the medullary concentrations of both

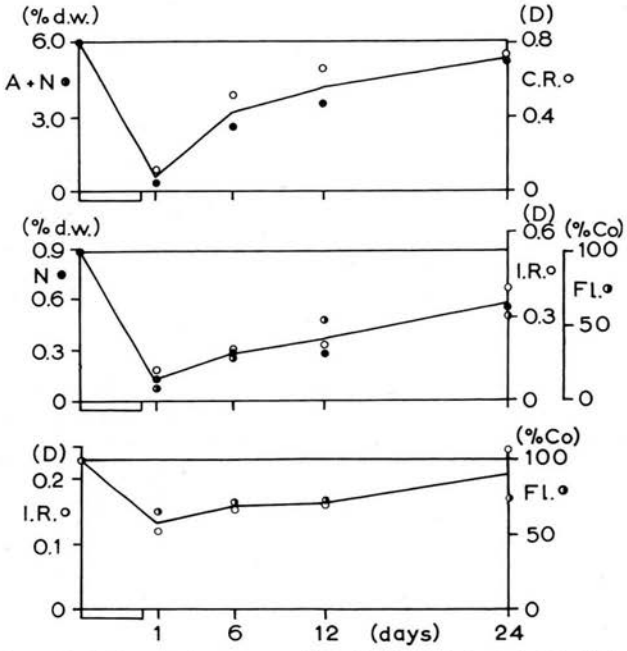


Fig. 1. Effect of reserpine on catecholamines of the adrenal medulla.

The injection period is indicated by a white rectangle below each graph (4 mg/kg daily for 4 days). The time scale is days after the last injection.

Top graph: Concentration of both catecholamines (A + N; full circles), expressed as % of dry weight of medulla, and intensity of the chromaffin reaction (C. R.; open circles), expressed in terms of optical density. Each circle represents the mean of 6—8 animals. The line has been drawn between the mean chemical and histochemical values.

Center graph: Concentration of noradrenaline (N; full circles), % of dry medullary weight; intensities of the iodate reaction (I. R.; open circles) in terms of optical density and of the formalin induced fluorescence (Fl.; half-filled circles), % of the corresponding mean control value. The intensities of the iodate reaction and the formalin-induced fluorescence were measured from the noradrenaline-containing cell islets.

Bottom graph: Intensities of the iodate reaction and the formalin-induced fluorescene in the medullary "background", *i. e.* adrenaline-storing cells. Symbols as in the center graph. Note that fluorescence is expressed as % of the corresponding (*i. e.* background) mean control value; therefore the fluorescence scales in this and the center graph are not comparable.

adrenaline and noradrenaline returned to normal within 4 days after the last injection. The chromaffin reaction, the iodate reaction and the intensity of the formalin-induced fluorescence were then similar of intensity and distribution as the corresponding reactions in the controls.

After daily injections of 4 mg/kg of reserpine during 4 consecutive days, pronounced effects were obtained. The results of the chemical analyses and of the quantitative measurements of the histochemical reactions are presented in Fig. 1. Each circle in each graph represents a mean value of a group of 6—8 animals, excepting the controls whose number was 26. The total number of animals in this experiment was 53. The injection period is indicated by a white rectangle below the base line. The scales of each graph have been adjusted so as to set the mean control values at the same height, which makes easier the comparison of the catecholamine concentrations and the intensities of the histochemical reactions.

The top diagram of Fig. 1 shows that there was an almost total loss of both

catecholamines 1 day after the last injection and that there was also a corresponding loss in the intensity of the chromaffin reaction, which actually was entirely negative in most sections, the residual loss of light being due to refraction rather than absorption (*cf.* ERÄNKÖ and RÄISÄNEN 1960).

Both the catecholamine concentration and the intensity of the chromaffin reaction returned slowly to normal. When these variables were expressed as % of the mean control value, and these relative values were compared, it was found that there was no statistically significant difference between the chemical and the histochemical data, although the mean density of the chromaffin reaction was somewhat higher 6 and 12 days after the last injection than the catecholamine concentration.

The center diagram of Fig. 1 shows corresponding changes in the noradrenaline concentration of the medulla and in the intensities of the iodate reaction and the formalin-induced fluorescence in the specific noradrenaline-containing medullary cell islets. There is a surprisingly good agreement between these variables, and it is clearly evident that in this experiment no increase over the control value in the medullary concentration of noradrenaline occurred during the restitution period. On the contrary, noradrenaline concentration tended to return more slowly towards normal than the total catecholamine concentration.

The bottom diagram illustrates the changes in the intensities of the iodate reaction and of the formalin-induced fluorescence in the medullary background, which consists of cells normally storing adrenaline. It is to be noted that the density and fluorescence scales are not the same as those in the center diagram, since again the mean control values have been placed at the same height from the zero line as in the two diagrams above.

No significant differences were found between the relative changes of these two reactions in the background, both showing first a decrease and then a slow return to normal. The relative decrease was smaller in these histochemical reactions than in the noradrenaline concentration but this is to a great part due to the facts that scattering results in a non-specific loss of light even in practically colourless sections and that there was some residual non-specific fluorescence in the medullary cells even after practically total loss of noradrenaline. There was no increase in the intensity of these histochemical reactions over the mean control value at any stage of the experiment.

Discussion

In our experiments 2 mg/kg of reserpine daily for three days caused a transient loss of catecholamines from the adrenal medulla but their concentration returned to normal within 4 days. Four consecutive daily injections of 4 mg/kg resulted in an almost complete loss of both adrenaline and noradrenaline, and recovery took several weeks.

In both experiments the concentrations of adrenaline and noradrenaline changed in parallel and in no instance was any absolute or relative increase above normal observed in the noradrenaline concentration. This is at variance with the results obtained by CALLINGHAM and MANN (1958a) and COUPLAND (1959). The former authors observed after 3 daily injections of 1mg/kg of reserpine first a decrease and after 3 days an increase in the noradrenaline content 300 % above the normal level. Adrenaline content also dropped first but it returned gradually to normal in the course of 2 weeks, during which time the temporarily elevated noradrenaline content slowly decreased to normal. In COUPLAND's (1959) experiment the noradrenaline content did not rise above the control level but it returned to normal before the adrenaline content; thus, the relative noradrenaline content temporarily increased. Moreover, COUPLAND (1959) made calculations suggesting that the noradrenaline content of those cells of the adrenal medulla which normally store mainly adrenaline was increased during the recovery phase.

Several factors must be considered in trying to explain the differences in the results obtained by CALLINGHAM and MANN (1958a), by COUPLAND (1959) and by us. First of all, the methods of catecholamine determination were different. CALLINGHAM and MANN (1958a) used rat's blood pressure and rat's uterus as bioassay techniques. These methods are subject to large errors when mixtures of adrenaline and noradrenaline are assayed (GADDUM and HOLZBAUER 1957). COUPLAND (1959) used a colorimetric method in which the differentiation of adrenaline and noradrenaline in a mixture is based on the fact that adrenaline is more readily oxidised at a low pH than noradrenaline. Also this method is subject to errors, particularly when much cortical extract, rich in ascorbic acid, and a relatively small amount of noradrenaline are present in the mixture, as is the case in the adrenal medulla of the rat. In our work the amines were first separated by paper chromatography, and thereafter an accurate fluorometric method of determination was used.

Fairly small errors in the estimation of the relative proportions of noradrenaline and adrenaline in COUPLAND's (1959) experiments would be sufficient to make non-significant the apparent small increase which he observed in the relative noradrenaline content during recovery. While COUPLAND's (1959) calculations seemed, furthermore, to suggest that there was an increase in the quantity of noradrenaline in the adrenaline-storing parts of the medulla, he did not histochemically observe any increase in the intensity of the iodate reaction in these areas. Even assuming that his figures for adrenaline and noradrenaline amounts were correct, the calculations which he made of the noradrenaline content of the medulla outside the specific cell islets are subject to criticism; they were based on measurements made by recording planimetrically the relative volume occupied by the noradrenaline-storing cell islets in the medulla, which he found decreased during the recovery.

Since the intensity of the iodate reaction was decreased and patchy in these cell islets during the recovery phase, COUPLAND (1959) himself found the delineation of the islets difficult and considered this in part responsible for the observed decrease in the volume of the islets after reserpine adminstration. Therefore, the apparently lower volume of the cell islets may indicate a temporary loss of noradrenaline from the noradrenaline-storing cells in the islets, rather than a real diminution in the size of the cell islets and an increase of noradrenaline concentration in the adrenaline-storing medullary cells outside the islets.

The observations made by COUPLAND (1959) and those of the present study are in agreement concerning the following points: reserpine caused in both studies a loss of both adrenaline and noradrenaline as well as a decrease in the intensities of the chromaffin and of the iodate reaction, and during the recovery phase there was no increase of the absolute noradrenaline content above the normal level. In both studies, furthermore, the intensity of the iodate reaction in the medullary background, *i. e.* in the cells normally storing adrenaline, did never increase above normal; indeed, our quantitative measurements indicated a decrease in this variable.

While COUPLAND's work and our differ, therefore, mainly in the interpretation, the results obtained by CALLINGHAM and MANN (1958a) are entirely different from those of COUPLAND and ours as far as changes in the noradrenaline content are concerned. It is difficult to find a pertinent explanation for this difference.

It does not seem likely that the very great increase which CALLINGHAM and MANN observed in the pressor response obtained with the extracts made from adrenals in the recovery phase could have been caused by errors in the assay. However, their evidence of the increase in the noradrenaline content is based on the assumption that the increase in the pressor response is due mainly to this amine. It is, however, not altogether impossible that the high assay values are in fact due to some other pressor substance whose concentration increases in the medulla during recovery.

Another possibility is that differences in the susceptibility of the rats towards reserpine in the different laboratories are of importance. Strain-dependent differences in the catecholamine loss after reserpine administration have earlier been demonstrated by COUPLAND (1958), who observed that Wistar rats required three times as much reserpine for an equal depletion as rats of the Sprague-Dawley strain. Dietary differences may also play a role in this respect.

Further studies are in progress on this problem in collaboration with Dr. K. R. BUTTERWORTH, who belongs to the same research group as Dr. MANN and Dr. CALLINGHAM.

This study has been supported by research grants from Suomen Kulttuurirahasto (Finland's Cultural Fund) and from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

References

CALLINGHAM, B. A. and M. MANN, Adrenaline and noradrenaline content of the adrenal gland of the rat following depletion with reserpine. *Nature* (Lond.) 1958a. *181.* 423—424.

CALLINGHAM, B. A. and M. MANN, Replacement of adrenaline and noradrenaline in the innervated and denervated adrenal gland of the rat, following depletion with reserpine. *Nature* (Lond.) 1958b. *182.* 1020—1021.

CAMANNI, F., O. LOSANA and G. M. MOLINATTI, Selective depletion of nor-adrenaline in the adrenal medulla of the rat after administration of reserpine (histochemical research). *Experientia* (Basel) 1958. *14.* 199.

CARLSSON, A. and N.-Å. HILLARP, Release of adrenaline from the adrenal medulla of rabbits produced by reserpine. *Kungl. Fysiogr. Sällsk. i Lund Förhandl.* 1956. *26.* Nr 8. 1—2.

COUPLAND, R. E., Strain sensitivity of albino rats to reserpine. *Nature* (Lond.) 1958. *181.* 930—931.

COUPLAND, R. E., The catechol amine content of the adrenal medulla of the rat following reserpine-induced depletion. *J. Endocr.* 1959. *18.* 154—161.

ERÄNKÖ, O., Histological sampling, chromatographic separation, and determination adrenaline and noradrenaline in the adrenal medulla. *Ann. Med. exp. Biol. Fenn.* 1954. *32.* 392—401.

ERÄNKÖ, O. and V. HOPSU, Effect of reserpine on the histochemistry and content of adrenaline and noradrenaline in the adrenal medulla of the rat and the mouse. *Endocrinology* 1958. *62.* 15—23.

ERÄNKÖ, O. and L. RÄISÄNEN, Cytophotometric study on the chromaffin reaction, the iodate reaction and formalin-induced fluorescence as indicators of adrenaline and noradrenaline concentrations in the adrenal medulla. *J. Histochem. Cytochem.* 1960. (In press.)

EULER, U. S. v. and I. FLODING, A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta physiol. scand.* 1955. *33.* Suppl. 118. 45—56.

GADDUM, J. H. and M. HOLZBAUER, Adrenaline and noradrenaline. *Vitam. and Horm.* 1957. *15.* 151—203.

HILLARP, N. Å. and B. HÖKFELT, Histochemical demonstration of noradrenaline and adrenaline in the adrenal medulla. *J. Histochem. Cytochem.* 1955. *3.* 1—5.

Stockholm 1961. Kungl. Boktr. P. A. Norstedt & Söner

Acta physiol. scand. 1961. 51. 247—253

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Distribution and Concentration of Adrenaline and Noradrenaline in the Adrenal Medulla of the Rat Following Depletion Induced by Muscular Work

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

Received 9 November 1960

Abstract

ERÄNKÖ, O. and M. HÄRKÖNEN. *Distribution and concentration of adrenaline and noradrenaline in the adrenal medulla of the rat following depletion induced by muscular work.* Acta physiol. scand. 1961. *51.* 247—253. — The rats were allowed to run for 16 hours, to rest for 12 hours and to run again for 10 hours. They were killed immediately, 3, 6, 12 or 19 days thereafter. Concentrations of adrenaline and noradrenaline in the adrenal medulla were measured chemically. The intensities of the chromaffin reaction, the iodate reaction and formalin-induced fluorescence in adrenal sections were quantitated by microphotometry. Running caused an almost complete loss of both catecholamines from the adrenal medulla and the histochemical reactions turned almost negative. The catecholamine concentrations and the histochemical reactions returned to normal in 6 days. During the recovery phase the behaviour of the noradrenaline-storing medullary cells differed histochemically from that of the adrenaline-storing cells.

Muscular work is known to be a potent stimulator of the adrenal medulla (*e. g.* EULER 1956, KÄRKI 1956). However, we are not aware of studies in which the concentrations in the adrenal medulla of both adrenaline and noradrenaline had been studied after long-lasting muscular work. HÖKFELT (1951) reported that after swimming for 30—90 min the noradrenaline content of rat adrenals showed a tendency to rise, whereas the adrenaline content

tended to decline. He did not study the effect of longer-lasting work. Since interesting features in the recovery of the catecholamines have been reported after depletion by pharmacological substances, *e. g.* reserpine (CALLINGHAM and MANN 1958 a, b, COUPLAND 1959, ERÄNKÖ and HOPSU 1961), we undertook a similar study using muscular work as a stimulus.

Material and Methods

Experimental

Adult albino rats were used. They were allowed to run in a rotating wire cylinder for varying periods. In preliminary experiments the effect on the adrenal medulla was investigated histochemical methods only, using the chromaffin reaction, the iodate reaction and formalin-induced fluorescence (see below) as indicators of the amount of the catecholamines in the medulla. In these experiments, a single period of work lasting up to several hours resulted only in a partial depletion of the medullary catecholamines, and a rapid restoration to normal occurred within a day after the work had been stopped.

However, when the rats were allowed to run for 16 hours, to rest thereafter for 12 hours and to run again for 10 hours, an almost total disappearance of catecholamines was observed, as indicated by the three histochemical reactions, which all turned almost negative. This experimental arrangement, which proved reproducible, was then adopted as a routine procedure, and the results to be reported in this paper have been obtained by using it.

The animals were killed by decapitation inmediately, 3, 6, 12 or 19 days after the end of the second running period, together with about the same number of similar but untreated control rats. Both adrenals were cut into two pieces of equal size. One piece was used for chemical analysis, the remaining 3 pieces for histochemical studies.

Chemical methods

Fresh adrenal sections cut at 50 μ were freeze-dried and pieces of pure dry medulla were dissected out and weighed. The catecholamines were either first separated by chromatography, eluted and determined separately by fluorometry after oxidation at pH 6 (*e. g.* EULER and FLODING 1955) or determined directly in the extract, making the fluorometric measurements at two exciting wavelengths, 366 mμ and 436 mμ for the determination of the relative proportion of noradrenaline in the mixture (*e. g.* COHEN and GOLDENBERG 1957). For further details of the method, see ERÄNKÖ and RÄISÄNEN (1960). Since the two methods gave comparable results, the data obtained with them were pooled and handled together.

Histochemical methods

One of the three remaining adrenal pieces was immersed in a mixture of potassium dichromate and formalin for the demonstration of both catecholamines by the chromaffin reaction, another was plunged into a saturated solution of potassium iodate for the demonstration of noradrenaline (HILLARP and HÖKFELT 1955), and the third one was fixed in a mixture of calcium chloride and formalin to develop the formalin-dependent fluorescence in the noradrenaline cell islets. Frozen sections cut at 50 μ were mounted in glycerol. For details see the previous paper (ERÄNKÖ and HOPSU 1961).

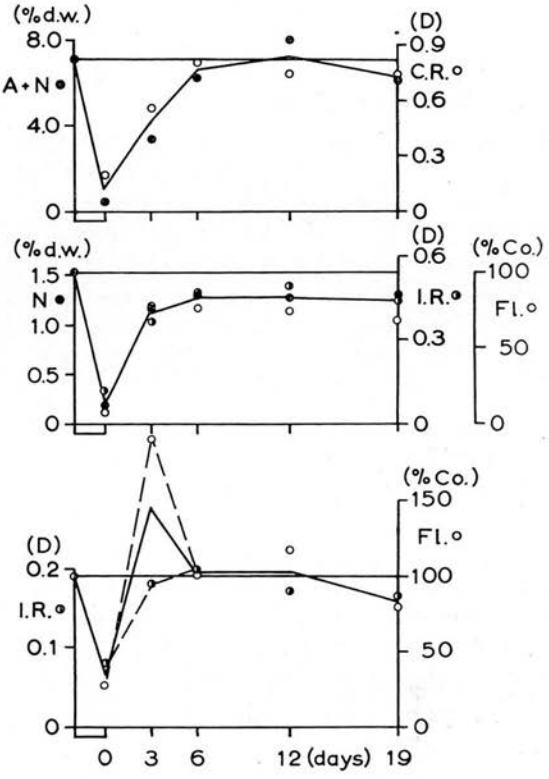


Fig. 1. Effect of muscular work on catecholamines of the adrenal medulla.

The running period is indicated by a white rectangle below the base line. Each circle represents the mean of a group of 5—7 animals. The solid line has been drawn between the mean chemical and histochemical values.

Top graph: Concentration of both catecholamines (A + N; full circles), % of dry medullary weight, and optical density (D) of the chromaffin reaction (C. R.; open circles).

Center graph: Concentration of noradrenaline (N; full circles), % of dry medullary weight; optical density (D) of the iodate reaction (I. R., half-filled circles) and intensity of the formalin-induced fluorescence (Fl.; open circles), % of the corresponding mean control value. The intensities of the iodate reaction and the fluorescence were measured from the noradrenaline-storing cell islets.

Bottom graph: Iodate reaction and formalin-induced fluorescence in the medullary "background", *i.e.* adrenaline-storing cells. Note that fluorescence is expressed as % of the corresponding (*i. e.* background) mean control value. Therefore the absolute values of fluorescence in this and the center graph are not comparable.

Quantitative measurement of histochemical reactions

The optical densities of the chromaffin reaction and the iodate reaction, as well as the intensity of the formalin-induced fluorescence were measured by microphotometry. The method has been described in detail elsewhere (ERÄNKÖ and RÄISÄNEN 1960, see also ERÄNKÖ and HOPSU 1961). Measurements of the intensities of the iodate reaction and the formalin-induced fluorescence were carried out separately on the noradrenaline-containing cell islets and on the medullary background consisting of cells normally storing mainly adrenaline.

Measurement of medullary volume

A separate experiment was carried out to study the effect of running on the volume of the adrenal medulla. The animals were killed immediately, 3 or 6 days after running in two consecutive days according to the schedule described earlier in this paper. One adrenal was immersed in the dichromate-formalin mixture, the other in a saturated iodate solution. After 24 hours, both glands were postfixed in 4 % formaldehyde. The medullary volume was planimetrically measured from a complete series of sections cut from the gland fixed in dichromate-formalin (ERÄNKÖ 1954).

Results

The results are presented in Fig. 1. Each circle represents a mean of a group consisting of 5—7 rats, excepting the controls, whose number was 28. The total number of animals in this experiment was 58. The running period is indicated by a white rectangle below the base line. The scales of each graph have been adjusted by setting the mean control values at the same height. The fluorescence intensity is expressed as percent of the corresponding mean control value.

The top diagram indicates a pronounced loss of catecholamines and a decrease in the intensity of the chromaffin reaction immediately after running. Both variables returned to normal in 6 days, and remained at the normal level thereafter. The changes in the catecholamine content and in the chromaffin reaction were parallel, and in no stage of the experiment was there any significant difference between their mean values.

The center diagram illustrates the changes in the noradrenaline concentration of the medulla and in the intensities of the iodate reaction and of the formalin-induced fluorescence in the specific cell islets normally storing noradrenaline. The changes in these variables are closely associated showing a pronounced loss of noradrenaline after running and a return to normal in a few days. Comparison of this diagram and the upper one seemingly suggests that the noradrenaline concentration was nearer the normal value three days after the running than was the total catecholamine concentration, which would indicate that the rate of formation of noradrenaline was higher than that of adrenaline. However, there was no statistically significant difference on the third day between the mean of the total catecholamine content expressed as percent of the control mean and that of the mean noradrenaline content expressed in the same way. Although the means of the three variables in the center diagram remained below the corresponding normal mean on the 6th, 12th and 19th day, the differences from the corresponding control means were not statistically significant.

The bottom diagram illustrates changes in the intensities of the iodate reaction and of the formalin-induced fluorescence in the medullary "background", *i. e.*, in the cells normally storing adrenaline. Because the scales have been adjusted by setting the mean control value, which is much smaller than in the cell islets, at the same height as in the two upper graphs, smaller absolute changes correspond to relative changes equal to those in the upper graphs.

Running caused a decrease in the intensities of the iodate reaction and the formalin-induced fluorescence in the background, and the values returned quickly towards normal. On the third day, the intensity of the iodate reaction was already at the control level. This is of interest because neither the noradrenaline concentration in the medulla nor the histochemical reactions in the noradrenaline cell islets had returned to normal during the same time.

Table I. Effect of running on the volume of the adrenal medulla

Treatment	Number of animals	Volume of medulla (per cent of controls)		P[1]
		Mean	SD	
Controls	8	100.0	9.6	—
Running, killed immediately....	9	126.4	19.1	~ 0.003
Running, killed after 3 days....	7	108.5	6.3	> 0.05

[1] The value of P has been calculated with the aid of the t-test and shows the significance of the difference from the control group.

The difference of the means of the intensity of the iodate reaction in the specific cell islets and that in the background, both expressed in terms of percent of the corresponding control mean, is statistically significant ($P \sim 0.02$).

The most striking feature in this graph is, however, the very high fluorescence intensity of the background on the 3rd day. This increase is statistically highly significant ($P < 0.001$). From the 6th day on the intensity of both the iodate reaction and the fluorescence kept near the corresponding control mean and did not differ from it significantly.

Table I shows the changes in the volume of the adrenal medulla immediately and 3 days after the last running period. Running caused a 26 percent increase in the medullary volume but after 3 days the mean volume did not any more differ significantly from that of the control group. The chromaffin reaction and the iodate reaction were almost negative immediately after running and their intensities were according to visual judgement about normal after 3 days' rest. However, after 3 days the intensity of the iodate reaction in the background of the medulla tended to be higher than in the controls.

Discussion

Long-lasting muscular work caused in the present study a pronounced depletion of both adrenaline and noradrenaline from the adrenal medulla. It is not possible to say in how far this depletion was due to muscular work alone and how much other factors such as general exhaustion, lack of sleep and diminished intake of food and water contributed to the effect observed. Fasting or lack of water for several days cause hardly any changes in the intensity of the chromaffin reaction in the adrenal medulla of the rat (ERÄNKÖ, unpublished) but they both may nevertheless potentiate the effect of muscular work. However, whatever the mechanism of the depletion is, it was reproducible and reversible.

The concentrations of both catecholamines and the intensities of the histochemical reactions employed returned to normal in 6 days. This period is much shorter than that required for recovery after reserpine administration, which resulted in a comparable initial loss of catecholamines from the adrenal medulla (*cf.* Fig. 1 in this paper with Fig. 1 in ERÄNKÖ and HOPSU 1961). Such a difference suggests that reserpine seriously interferes with the synthesis and/or storage of adrenaline and noradrenaline in the adrenal medulla, while even prolonged muscular work has less or no such effect. This fits well in with the recent observations demonstrating that reserpine prevents the storage of catecholamines in the cytoplasmic granules (BERTLER, HILLARP and ROSENGREN 1960).

In general, the results of the chemical determinations agreed in the present study well with those obtained by quantitating the histochemical reactions. Likewise, the changes in the adrenaline and noradrenaline concentrations were parallel. In these respects the results were similar as those obtained in the reserpine experiment (ERÄNKÖ and HOPSU 1961).

However, 3 days after the end of the running period, *i. e.* when an active resynthesis of the depleted amines took place, an interesting divergence was observed between the intensities of the iodate reaction and the formalin-induced fluorescence in the medullary background: while the intensity of the iodate reaction was equal to that in normal controls, the intensity of the fluorescence was almost twice the normal value.

This shows that the formalin-induced fluorescence, although it usually is parallel with the iodate reaction, is not always so. For this there are three possible explanations. First, the formalin-induced fluorescence may not, like the iodate reaction, depend, at least exclusively, on noradrenaline but to some other substance whose concentration tends usually but not always to vary parallelly with that of noradrenaline. Second, the increase in the intensity of the fluorescence is perhaps not due to an increase in the amount of the fluorescent substance but may be caused by some other change, *e. g.* by a loss of a substance quenching the fluorescence; this explanation is compatible with the assumption that noradrenaline is responsible for the fluorescence. Third, the iodate reaction may not demonstrate noradrenaline outside the granules.

Of these alternatives the first one appears most likely but the nature of the postulated fluorescent substance, if other than noradrenaline, remains obscure. At any rate, the observation completes two earlier ones in which a discrepancy was observed between the intensities of the fluorescence and the iodate reaction (ERÄNKÖ 1955, ERÄNKÖ, HOPSU and RÄISÄNEN 1959).

On the 3rd day, the intensity of the iodate reaction in the medullary background was already normal although the total catecholamine concentration and the noradrenaline concentration were still below normal. Assuming that the iodate reaction is a faithful indicator of the local noradrenaline concentra-

tion, this means that the rate of formation of noradrenaline in the normally adrenaline-storing cells was higher than the rate of the subsequent methylation to noradrenaline. It is noteworthy that CALLINGHAM and MANN (1958 a, b) and COUPLAND (1959) arrived at the same conclusion in experiments in which the loss of catecholamines was induced by reserpine. However, since the results obtained in this laboratory with reserpine (ERÄNKÖ and HOPSU 1961) are different and since the matter is under investigation in collaboration with the London laboratory, it seems better to postpone the discussion of this matter until the results of the joint study are available.

This study has been supported by research grants from Suomen Kulttuurirahasto (Finland's Cultural Fund) and from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

References

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN, Storage of new-formed catecholamines in the adrenal medulla. *Experientia* (Basel) 1960. *16.* 419—420.

CALLINGHAM, B. A. and M. MANN, Adrenaline and noradrenaline content of the adrenal gland of the rat following depletion with reserpine. *Nature* (Lond.) 1958 a. *181.* 423—429.

CALLINGHAM, B. A. and M. MANN, Replacement of adrenaline and noradrenaline in the innervated and denervated adrenal gland of the rat, following depletion with reserpine. *Nature* (Lond.) 1958 b. *182.* 1020—1021.

COHEN, G. and M. GOLDENBERG, The simultaneous fluorimetric determination of adrenaline and noradrenaline in plasma. *J. Neurochem.* 1957. *2.* 58—70.

COUPLAND, R. E., The catechol amine content of the adrenal medulla of the rat following reserpine-induced depletion. *J. Endocr.* 1959. *18.* 154—161.

ERÄNKÖ, O., Estimation of the volumes of the cortex and medulla from adrenal sections. *Ann. Med. exp. Biol. Fenn.* 1954. *32.* 377—391.

ERÄNKÖ, O., Histochemistry of noradrenaline in the adrenal medulla of rats and mice. *Endocrinology* 1955. *57.* 363—368.

ERÄNKÖ, O. and V. HOPSU, Distribution and concentration of adrenaline and noradrenaline in the adrenal medulla of the rat following reserpine-induced depletion. *Acta physiol. scand.* 1961. *51.* 239—246.

ERÄNKÖ, O. and L. RÄISÄNEN, Cytophotometric study on the chromaffin reaction, the iodate reaction and formalin-induced fluorescence as indicators of adrenaline and noradrenaline concentrations in the adrenal medulla. *J. Histochem. Cytochem.* 1960. (In press).

ERÄNKÖ, O., V. HOPSU and L. RÄISÄNEN, Effect of prolonged administration of nicotine on the medullary volume and the distribution of noradrenaline in the adrenals of the rat, the mouse and the guinea pig. *Endocrinology* 1959. *65.* 293—297.

EULER, U. S. v., Noradrenaline, Chemistry, Physiology, Pharmacology and Clinical Aspects. Springfield, Ill., Charles C. Thomas. 1956. pp. 382.

EULER, U. S. v. and I. FLODING, A fluorimetric micromethod for differential estimation of adrenaline and noradrenaline. *Acta physiol. scand.* 1955. *33.* Suppl. 118. 45—56.

HILLARP, N.-Å. and B. HÖKFELT, Histochemical demonstration of noradrenaline and adrenaline in the adrenal medulla. *J. Histochem. Cytochem.* 1955. *3.* 1—5.

HÖKFELT, B., Noradrenaline and adrenaline in mammalian tissues. *Acta physiol. Scand.* 1951. *25.* Suppl. 92. 1—134.

KÄRKI, N., The urinary excretion of noradrenaline and adrenaline in different age groups, its diurnal variation and the effect of muscular work on it. *Acta physiol. Scand.* 1956. *39.* Suppl. 132. 1—96.

Stockholm 1961. Kungl. Boktr. P. A. Norstedt & Söner

ACTA ENDOCRINOLOGICA
39 (1962) 288–293

Department of Anatomy, University of Helsinki,
Siltavuorenpenger, Helsinki, Finland

HISTOCHEMICALLY DEMONSTRABLE CHOLINESTERASES IN THE ADRENAL MEDULLA OF THE HAMSTER AND THE EFFECT OF DENERVATION

By

O. Eränkö, V. Hopsu and A. Palkama

ABSTRACT

Acetylcholinesterase and non-specific cholinesterase gave positive histochemical reactions in the nerve fibres of the adrenal medulla of the hamster. Acetylcholinesterase activity was also observed in the fine nervous network covering the whole medulla, while non-specific cholinesterase activity was limited to fewer fibres. Division of the splanchnic nerve did not essentially affect the non-specific cholinesterase reaction but abolished most of the acetylcholinesterase activity. However, some fibres both in the cortex and in the medulla retained their acetylcholinesterase activity after denervation.

In a previous study (*Eränkö* 1959) it was observed that acetylcholinesterase (AChE) has a distribution slightly different from that of non-specific cholinesterase (nsChE) in the adrenal medulla of the rat. Furthermore, division of the splanchnic nerve causes an almost total loss of the AChE activity but has little influence on the nsChE activity of the medullary fibres (*Eränkö et al.* 1959). In the present paper similar studies on cholinesterases of the hamster adrenal are described.

METHODS

Experimental procedure

Adult golden hamsters were unilaterally denervated by dividing the left splanchnic nerve under the diaphragm. The animals were killed by decapitation about 2 weeks after the denervation. Both adrenals were removed and fixed in calcium formol for 2–4 hours. Frozen sections cut at 20 μ were rinsed in physiological saline or water and allowed to dry on slides, mounting on the same slide sections from both the left, denervated, and the right, intact, adrenal of each animal.

Histochemical techniques

Gomori's (1952) modification of *Koelle*'s (1951) thiocholine method was employed for the demonstration of cholinesterase, using acetylthiocholine and butyrylthiocholine as substrates. Eserine, 62.C.47 (1:5-*bis*-(4-trimethylammoniumphenyl)pentan-3-one diiodide) (*Burgen* 1949) and *iso*-OMPA (tetra*iso*propylpyrophosphoramide) (*Aldridge* 1953) were used as inhibitors (see also *Pepler & Pearse* 1957). The sections were first incubated for 20–30 min in the inhibitor solutions and thereafter with the substrate in the presence of the same inhibitor concentration.

For the chromaffin reaction, adrenal halves were fixed in a mixture of 1 volume of 35 per cent formaldehyde and 19 volumes of 3.5 per cent potassium dichromate. The iodate reaction was carried out according to *Hillarp & Hökfelt* (1955). Formalin-induced fluorescence was studied as described previously (*Eränkö* 1955).

RESULTS

1. Normal adrenals

Nervous structures

The nerves reach the adrenal medulla through a large area of the cortex, which is penetrated by numerous straight nerve bundles separated from each others by nerve-free cortical tissue. Before entering the cortex the nerves send fibres to a narrow subcapsular network. There are also solitary fibres in the cortex, straight ones directed towards the medulla and, especially near the cortico-medullary junction, tortuous, irregularly running fibres. In the medulla there is a rich nervous network. Some of these fibres are thicker and surround the medullary cell acini and the secretory cells in them. The others are finer and form another, finemeshed network which covers the whole medulla. Along these fine fibres, small oval or round bodies are visible.

Histochemical observations

Acetylthiocholine was used as a substrate, always together with 10^{-6} M *iso*-OMPA, a combination which should selectively demonstrate AChE (see *Pepler & Pearse* 1957; *Eränkö* 1959). For a presumably selective demonstration of nsChE, butyrylthiocholine and 10^{-5} M 62.C.47 were used. In the following description it is assumed that AChE and nsChE are thus differentiated from each other. Positive reactions were obtained with both of these substrate-inhibitor combinations, while the histochemical reaction was almost completely inhibited when both *iso*-OMPA and 62.C.47 were used together. However, a faint reaction was seen in many medullary fibres after incubation in a solution containing acetylthiocholine, butyrylthiocholine, 10^{-6} M *iso*-OMPA and 10^{-5} M 62.C.47 or, instead of these two inhibitors, 10^{-6} M eserine. Since this residual reaction was very weak, it was evident that the reaction observed by using either of the two above-mentioned substrate-inhibitor combinations was mainly due to true cholinesterase activity.

All the nervous structures in the cortex and both coarse and fine fibres in the medulla showed a distinct AChE activity (Fig. 1). In addition, the cytoplasm of the parenchymal cells in the medulla showed a reaction which was much less intense. Peripheral areas of the medulla were somewhat more intensely stained than those in the centre, apparently mainly because the nerve fibres were both more numerous and more reactive in the regions of the peripheral cell acini.

With butyrylthiocholine and 62.C.47, numerous nerve fibres exhibited a positive nsChE reaction (Fig. 3). However, there were fewer positive fibres, most of them coarse, both in the cortex and the medulla. On the other hand, the cytoplasm of the cortical cells in the zona glomerulosa, which remained entirely negative with acetylthiocholine, was slightly but reproducibly positive, while, conversely, the cytoplasm of the medullary cells remained negative.

The pattern formed by the medullary fibres exhibiting a positive nsChE reaction was somewhat different from that of AChE positive fibres (compare Figs. 1 and 3). In the peripheral areas of the medulla the nsChE positive fibres formed loops, presumably enclosing the parenchymal cells, but the close network formed by the fine fibres demonstrable with acetylthiocholiae was not visible. The difference between the sections treated with acetylthiocholine and butyrylthiocholine was particularly clear in the central parts of the medulla. With acetylthiocholine a fine network of fibres was demonstrated there, while large areas were devoid of activity in the corresponding areas of sections incubated with butyrylthiocholine (compare lower left corners of Figs. 1 and 3).

Fig. 1.

AChE in the right intact adrenal. A strong reaction is seen in the fibres enclosing the parenchymal cells which form the peripheral medullary cell acini. The reaction is less intense in the centre of the medulla (below left), where fewer coarse fibres are present but a tight meshwork of fine positive fibres is visible. Several positive fibres can be distinguished in the cortex.

Fig. 2.

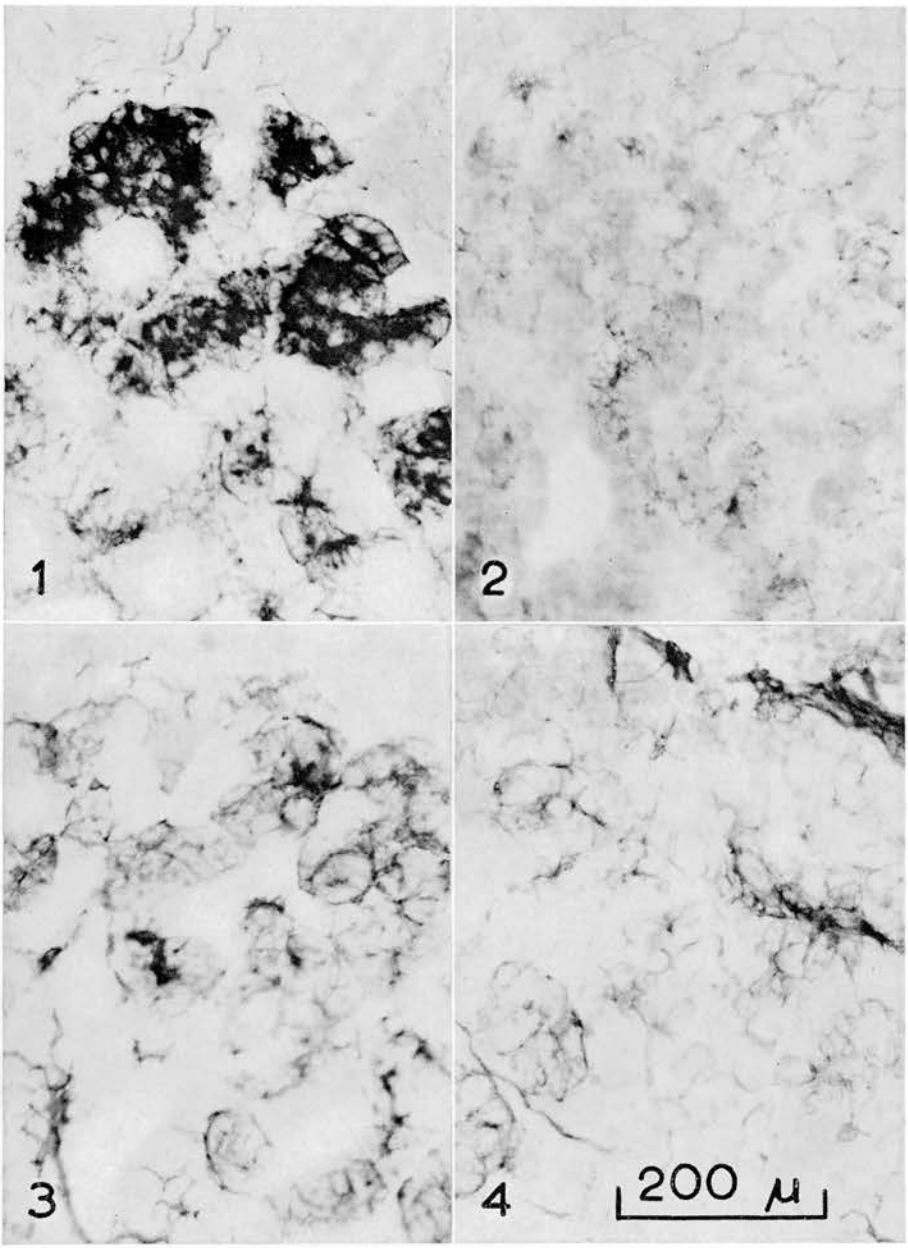
AChE in the left denervated adrenal of the same hamster. A great loss of activity is evident but some fibres are visible, both in the cortex and medulla. The photographic print has been developed somewhat longer than that in Fig. 1 to make visible the slightly positive reaction in the cytoplasm of the medullary cells.

Fig. 3.

nsChE in the right intact adrenal. Positive fibres are more frequent in the region of the peripheral medullary cell acini (up right) than in the central acini (lower left corner).

Fig. 4.

nsChE in the left, denervated adrenal. There is but little loss of reactive fibres and the intensity of the reaction is not much altered. Note the two cell acini above the number 4 with a positive fibre network.



Legends to the figures.

Figs. 1–4 are of the adrenal glands of the hamster. A narrow strip of cortex is seen in the upper margin of all figures. Below the corticomedullary junction is the peripheral zone of the medulla, containing in the hamster the noradrenaline-storing medullary cell acini. The central part of the medulla, composed of adrenaline-containing parenchymal cells, is visible in the lower part of each figure. Magnification in all figures is exactly the same, × 135.

II. Effect of denervation

Denervation caused a considerable loss of the AChE reaction, most of the previously positive fibres having either disappeared or became negative. However, some clearly positive fibres were still visible both in the cortex and in the medulla (Fig. 2). These fibres had an irregular and tortuous course, and large areas in the medulla were entirely devoid of them. Ganglion cells are apparently rare in the adrenal medulla of the hamster but fibres originating from the few nerve cells observed in the denervated adrenals gave a positive AChE reaction. The cytoplasm of the medullary parenchymal cells remained positive after denervation.

The distribution of nsChE activity was, on the contrary, little affected by denervation. Positive fibres of the same type and distribution as those in the intact adrenals were also seen in the denervated glands (Fig. 4), and it was not possible to observe with certainty even a weakening in the intensity of the reaction in the fibres.

The chromaffin reaction was positive throughout the medulla both in intact and in denervated adrenals, and no distinct differences in the intensity of the reaction were observed after denervation. The same also applied to intensities of the iodate reaction and of formalin-induced fluorescence, which equally well demonstrated the presence of noradrenaline-containing cell islets in the periphery of the medulla (*Eränkö* 1955) before and after denervation. Comparison of the distribution of fluorescence and the cholinesterase reactions in the same section showed that the fluorescent medullary cell islets in intact adrenals are covered by a richer network of both AChE and nsChE positive fibres than the adrenaline-containing central part of the medulla.

DISCUSSION

The results obtained are principally similar with those previously reported on rat adrenals (*Eränkö* 1959; *Eränkö et al.* 1959), as far as the different distributions of AChE and nsChE and their different responses to denervation are concerned. This is in agreement with the view that AChE is the enzyme directly concerned with the secretory innervation, while nsChE apparently plays a less important role in this respect.

It is of interest that although the number of ganglion cells seems to be smaller in the adrenal medulla of the hamster, more AChE positive fibres can be seen two weeks after denervation, as compared with the rat. The morphological characteristics of these fibres suggest that they do not belong to the main secretory innervation apparatus of the medulla. Similar fibres were also seen in intact adrenals, especially near the corticomedullary junction, while in the medulla their presence was difficult to recognise owing to the dense network of secretory fibres. It might be that the denervation-resistant AChE positive

fibres take part in the control of adrenal circulation (*cf. Harrison & Hoey* 1960) but there is little evidence to support this view.

Other problems concerning adrenal innervation also await clarification. By improving the localising power of the histochemical techniques it should be possible to decide finally, whether the medullary fibres exhibiting a positive nsChE reaction are, as seems likely, identical with the coarser fibres which show a high AChE activity. The termination and relation of the fine AChE positive but nsChE negative fibre network to the parenchymal cells also needs clarification. We have made efforts to investigate these problems using recent histochemical methods reported suitable for the localization of cholinesterase activity even at submicroscopic level (*Barrnett & Palade* 1959; *Lehrer & Ornstein* 1959). Unfortunately, these methods proved unsuitable for the study of the adrenal medulla, which did not give a positive reaction with either method.

REFERENCES

Aldridge W. N.: Biochem. J. *53* (1953) 62.
Barrnett R. J. & Palade G. E.: J. biophys. biochem. Cytol. *6* (1959) 163.
Burgen A. S. V.: Brit. J. Pharmacol. *4* (1949) 219.
Eränkö O.: Acta endocr. (Kbh.) *18* (1955) 174.
Eränkö O.: Histochemie *1* (1959) 257.
Eränkö O., Hopsu V. & Räisänen L.: J. Neurochem. *4* (1959) 264.
Gomori G.: Microscopic Histochemistry. University of Chicago Press, Chicago, Illinois (1960).
Harrison R. G. & Hoey M. J.: The Adrenal Circulation. Blackwell Scientific Publications, Oxford (1960).
Hillarp N.-Å. & Hökfelt B.: J. Histochem. Cytochem. *3* (1955) 1.
Koelle G. B.: J. Pharmacol. exp. Ther. *103* (1951) 153.
Lehrer G. M. & Ornstein L.: J. biophys. biochem. Cytol. *6* (1959) 399.
Pepler W. J. & Pearse A. G. E.: J. Neurochem. *1* (1957) 193.

Received on June 19th, 1961.

Histochemie 2, 383—388 (1962)

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki

HISTOCHEMICAL EVIDENCE OF THREE TYPES OF ESTERASES IN THE ADRENAL MEDULLA OF THE RAT

By

OLAVI ERÄNKÖ, AULIKKI KOKKO and ULLA SÖDERHOLM

With 6 Figures in the Text

(Received November 13, 1961)

Observations on histochemically demonstrable esterases in the adrenal medulla of the rat have been described in previous papers (ALLEN, ERÄNKÖ and HUNTER 1958; COUPLAND and HOLMES 1958; ERÄNKÖ 1959), in which the main interest was focussed on cholinesterases. On the other hand, esterase activity detectable in extracts of the adrenal medulla with the aid of starch gel electrophoresis has been found entirely (ALLEN et al. 1958) or predominantly (ERÄNKÖ, KOKKO and SÖDERHOLM 1962) eserine resistant and thus not due to cholinesterases. The present paper deals with observations made on adrenal esterases in sections employing the same combinations of substrate, coupling agent and inhibitor as used in the electrophoretic study (ERÄNKÖ et al. 1962).

Material and Methods

Adult albino rats, descendants of the Sprague-Dawley strain, were used. The animals were killed by decapitation, and the adrenals were removed immediately thereafter. The glands were usually fixed in calcium formol (1 volume of 35% formaldehyde stored over calcium carbonate, 6 volumes of 2% calcium chloride and 3 volumes of distilled water) for 2—4 hours at 20° C or overnight at 4° C. Frozen sections cut at 15—20 microns were rinsed in distilled water, floated on clean slides and allowed to dry. Some studies were carried out with fresh frozen sections, which were either allowed to thaw and dry on the slide or inserted in the end of a starch slab, subjected to the voltage gradient employed in the starch gel electrophoresis (see ERÄNKÖ et al. 1962) and treated with a slice from the end of the starch slab through the histochemical procedure together with the fixed and fresh sections attached on slides. It was thus hoped to obtain an idea of the solubilities of the different types of esterases in the sections.

Alpha-naphthyl acetate and alpha-naphthyl butyrate served as substrates. They were stored as 2% stock solutions made in acetone. The substrate mixture was prepared fresh each time by adding 2.5 ml of either stock solution into 50 ml of 0.1 M phosphate buffer, p_H 7.4. Blue RR Salt (National Aniline Division, New York) served as a coupling agent; it was found superior to the Red B Salt previously employed for this purpose (ALLEN et al. 1958, ERÄNKÖ 1959), as indicated by shorter incubation times and a cleaner "background". The diazonium salt was added dry into the substrate mixture in a concentration of about 10 mg/ml, and mixed by agitation. The substrate-coupler mixture was then filtered directly on the sections, and it was each minute replaced by a newly filtered mixture. Development of the reaction was followed under the microscope.

Eserine and E. 600 (p-nitrophenol diaethyl phosphate) were used as inhibitors, both in a concentration of 10^{-5} M. When either inhibitor was used, the section was first pre-incubated in an inhibitor solution made in the 0.1 M phosphate buffer and after 20—30 minutes treated with the mixture of substrate and coupler, into which the same concentration (10^{-5} M) of the same inhibitor had been added. Parallel controls were at the same time treated identically in solutions similar save for the absence of any inhibitor.

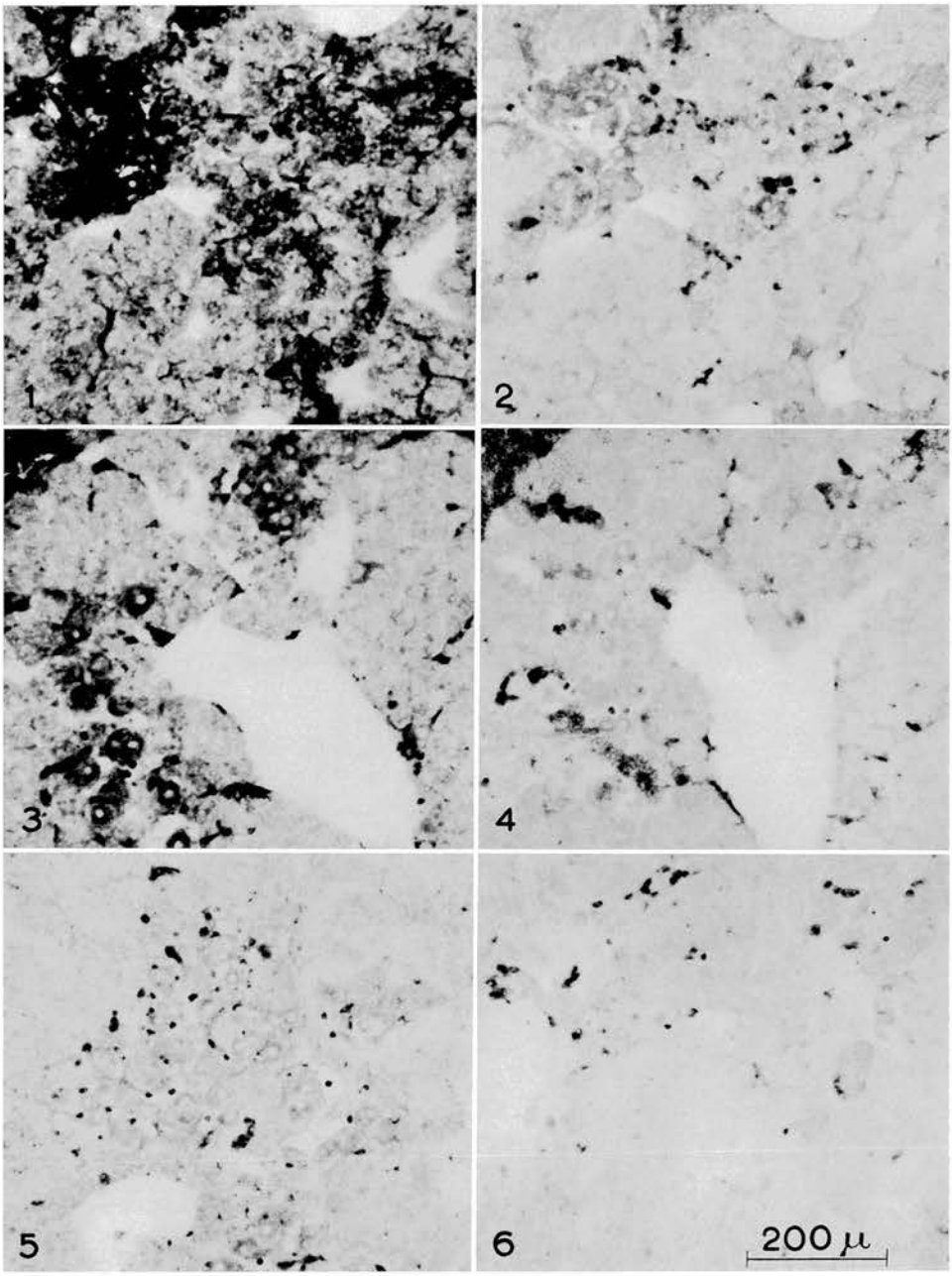


Fig. 1. Esterases of the adrenal medulla demonstrable with alpha-naphthyl acetate as a substrate. A strongly positive symphathetic ganglion in the upper left corner. A meshwork of positive fibres in the region of noradrenaline-containing cell islets is visible in the upper right corner, to be compared with the area shown in the lower left part of the figure

Fig. 2. The neighboring section to that shown in Fig. 1. Esterases demonstrable with alpha-naphthyl butyrate as a substrate. Note the less intense reaction in the nervous elements and the strongly positive small cells. Cytoplasm of the noradrenaline-containing cells in the right upper part of the figure is more intensely stained than that in the adrenaline-containing cells in the lower left area

Results

1. alpha-Naphthyl acetate. When no inhibitor had been used, a widely distributed reaction was obtained (Fig. 1). Nerve fibres and cells exhibited a very strong activity. They became positive in less than a minute and covered the sympathetic ganglia of the medulla with a dense deposit of dye during the period of 6 minutes, which was sufficient to bring about a positive reaction in other, less reactive medullary structures. Strongly positive fibres were especially numerous in the region of the noradrenaline-containing cell islets (see ERÄNKÖ 1959); fewer positive fibres were seen in the regions of the adrenaline-containing cells. Although the strong reaction in the nervous elements tended to cover other, less intensely reacting structures, it was possible to detect small, oval, strongly positive cells, which were neither ganglion cells nor chromaffin cells, scattered especially in the regions of the ganglia and the noradrenaline-containing cell islets. As yet it has not been possible to identify these cells with any recognized cell type. Therefore, they are simply referred to as small oval cells. A weak but clearly positive reaction was seen in the cytoplasm of all medullary cells. All cortical cells were strongly positive.

2. alpha-Naphthyl butyrate. The activity towards butyrate was much weaker than that towards acetate. Although the incubation time was prolonged to 15—20 minutes, the over-all staining of sections was less intense (Fig. 2). The distribution of the reaction was also clearly different. The nerve fibres reacted hardly at all, and the intensity of the reaction in the cytoplasm of the ganglion cells was lower than that observed with acetate. The noradrenaline cell islets were weakly but somewhat more intensely stained than the main background of the medulla consisting of adrenaline-containing cells. Although this difference in staining may indicate a higher level of esterase activity in the noradrenaline-containing cells, it must be pointed out that fixation in formalin makes these cells capable of giving a positive diazo reaction even in the absence of any substrate (ERÄNKÖ 1960). Against the relatively weakly positive background, small oval, strongly positive cells, predominantly situated in the ganglia and the noradrenaline-containing cell islets, were easy to recognize. Apparently these were the same cells which were strongly positive also when acetate was used as a substrate. The cortical cells were strongly positive but the intensity of the small oval cells was somewhat stronger.

3. Effect of eserine. With alpha-naphthyl acetate as a substrate, eserine (10^{-5} M) had a powerful inhibitory action on the reaction in the nerve fibres

Fig. 3. Esterases demonstrable with alpha-naphthyl acetate and eserine. A small area of cortex in the upper left corner. In spite of eserine, positive reactions are seen in the small oval cells, the ganglion cells and secretory medullary cells but nerve fibres are negative

Fig. 4. The neighboring section to that shown in Fig. 3. Eserine and alpha-naphthyl butyrate. The same components are positive as those in Fig. 3, although the intensity of the reaction is lower. Note that the small cells are more strongly positive than the cortical cells visible in the upper left corner and as a row of cells in the lower left part of the figure

Fig. 5. Esterases demonstrable with alpha-naphthyl acetate and E. 600. A medullary ganglion is in the centre. The intensity of the reaction is low in all other structures but the small cells. To be compared with Fig. 3

Fig. 6. Reaction obtained with alpha-naphthyl butyrate and E. 600. The small oval cells are strongly positive, the rest of the medulla almost negative. Magnification in all figures the same, as indicated by the scale mark

and cells (Fig. 3). While a distinct reaction was still discernible in the cytoplasm of the ganglion cells, the nerve fibres had virtually lost their activity. As a consequence, a picture much resembling that obtained with butyrate without inhibitor was seen (compare Figs. 2 and 3). In both cases, the small oval cells were the most intensely positive element (Figs. 3 and 4). However, with acetate and eserine the intensity of the reaction in the ganglion cells and in the parenchymal medullary cells was stronger than that in sections incubated with butyrate alone. With either substrate, the cortical cells retained most of their activity in spite of eserine.

4. Effect of E. 600. As can be expected, E. 600 had an even more powerful inhibitory effect than eserine. Both with acetate and with butyrate, the small oval cells, whose activity was but little affected, were almost selectively demonstrated in the medulla (Figs. 5 and 6). Some loss of activity occurred even in these cells, however, and it was possible to see that a round area in the cytoplasm near the nucleus was the main site of activity. Much activity remained also in the cortical cells, which were nevertheless less strongly positive than in sections incubated without inhibitor. The small oval cells were darker than the cortical cells, which seems to exclude the possibility that they are of cortical origin. A weakly positive reaction was observed in the ganglion cells and the parenchymal cells in the presence of E. 600, especially with acetate as a substrate.

5. Effect of electrophoresis. Fresh sections attached to slides and, especially, fresh sections subjected to electrophoresis were histologically poorly preserved after the histochemical procedure. However, comparison with formalin-fixed sections, treated identically according to the same procedure, showed that at least most of the esterase activity had remained in the section even after electrophoresis. This applied equally to the eserine sensitive, eserine resistant but E. 600 sensitive, and E. 600 resistant components of esterase activity.

Discussion

In agreement with previous observations (ALLEN et al. 1958, ERÄNKÖ 1959) it was observed that a major part of the esterase activity of the adrenal medulla detectable histochemically alpha-naphthyl acetate as a substrate is due to cholinesterases. This eserine-sensitive activity was responsible for practically all activity in the nerve fibres and for most activity in the nerve cells. It is of interest to note that alpha-naphthyl butyrate is a much less suitable substrate for cholinesterases but better adapted for the study of eserine-resistant esterases. It is therefore understandable that the intensity of the reaction obtained with this substrate in the secretory medullary cells, known to possess a weak but clear cholinesterase activity (ALLEN et al. 1958, ERÄNKÖ 1959), was weaker and less affected by eserine than that observed with alpha-naphthyl acetate.

Since eserine in the concentration employed in the present study was capable of abolishing the reaction in the nerve fibres, whose reaction was strongest of all medullary components in sections incubated with alpha-naphthyl acetate, it is reasonable to assume that all of the activity seen in sections treated with eserine is due to esterases other than cholinesterases. Such eserine resistant activity was strongest in the small oval cells but it was also present in the cytoplasm of the ganglion cells and the secretory medullary cells. E. 600 alone

inhibited most of this activity in the ganglion and secretory cells but affected little the strong reaction in the small oval cells.

After the considerations presented, the following conclusions seem justified: (1) nerve fibres in the medulla have a high cholinesterase activity but little or no other esterase activity; (2) the ganglion cells possess both cholinesterase activity and eserine resistant esterase activity, most of which is E. 600 sensitive; (3) a part of the weak activity in the secretory cells is due to cholinesterase, the rest being eserine resistant but E. 600 sensitive; (4) the small oval cells have a strong esterase activity, most of which is resistant to eserine and E. 600. The histochemical observations thus present evidence of at least three types of esterases in the adrenal medulla.

Taking into account the intensity of the reaction in, and the relative amounts of, the various medullary components, it can be assessed that most of the total activity demonstrable in medullary sections was due to cholinesterases, while most of the remaining activity was eserine resistant but E. 600 sensitive, the E. 600 resistant activity being limited to strongly positive but few cells, thus representing only a small part of the total esterase activity in the medulla. Because the same types of activities were essentially undiminished demonstrable also in fresh sections subjected to the electrophoretic procedure, it seems that the same esterases are relatively firmly attached to the medulla even without fixation in formalin.

On the other hand, esterases demonstrable with the same combinations of substrates, coupler and inhibitors by means of starch gel electrophoresis can be equally seen in the zymograms, whether repeatedly frozen and thawn medullary extracts or fresh sections are used as material inserted into the starting end of the starch slab (Eränkö et al. 1962). Accordingly, these esterases must be relatively soluble and they are therefore likely to leave the sections during the histochemical procedure. If this is true, as it seems, also concerning formalin-fixed tissues, one comes to the conclusion that histochemical techniques demonstrate desmo-enzymes, which may be different from the lyo-enzymes which can be separated by gel electrophoresis.

This conclusion is significantly supported by the fact that a major part of the esterase activity in medullary sections is due to cholinesterases, while cholinesterases are hardly demonstrable in the starch slabs after electrophoresis, bands exhibiting a strong, eserine-resistant but E. 600 sensitive reaction being clearly dominant (Eränkö et al. 1962). Even in the case that it may be possible to demonstrate similar types of enzyme activity, say eserine resistant but E. 600 sensitive esterase activity, both in sections and in starch slabs after electrophoresis, either technique is likely to give an incomplete picture of the total pattern of enzyme activities in living cells and tissues.

Summary

Esterases of the adrenal medulla have been studied histochemically using alpha-naphthyl acetate and butyrate as substrates, Blue RR Salt as a coupler and eserine and E. 600 as inhibitors. Three types of esterase activity were thus demonstrated: (1) cholinesterase activity in the nerve fibres, ganglion cells and secretory medullary cells; (2) eserine resistant but E. 600 sensitive esterase

activity in the ganglion cells and secretory cells; (3) E. 600 resistant activity in strongly positive, unidentified cells scattered in the medulla. The histochemical picture was essentially similar in sections of formalin-fixed tissue and in fresh sections subjected to the voltage gradient employed for electrophoretic separation of esterases. It is concluded that esterases histochemically demonstrable in sections are desmo-enzymes and at least to a major part different from the lyo-enzymes which can be separated by starch gel electrophoresis.

Acknowledgements. The authors are very grateful to Dr. Robert L. Hunter, Department of Anatomy, University of Michigan, for valuable advice and for samples of alpha-naphthyl butyrate and Blue RR Salt. Technical assistance by Mrs. Liisa Räisänen throughout the study is gratefully acknowledged.

This work has been supported by grants from the Sigrid Jusélius Foundation, Helsinki, and from the National Institute of Arthritis and Metabolic Diseases, the U.S. Public Health Service (A-1725).

References

Allen, J. M., O. Eränkö and R. L. Hunter: A histochemical study of the esterases of the adrenal medulla of the rat. Amer. J. Anat. **102**, 93—110 (1958).

Coupland, R. E., and R. L. Holmes: The distribution of cholinesterase in the adrenal glands of the rat, cat and rabbit. J. Physiol. (Lond.) **141**, 97—106 (1958).

Eränkö, O.: Specific demonstration of acetylcholinesterase and non-specific cholinesterase in the adrenal gland of the rat. Histochemie **1**, 257—267 (1959).

— Cell types of the adrenal medulla. Ciba Foundation Symposium on Adrenergic Mechanisms, edit. J. R. Vane, G. E. W. Wolstenholme and M. O'Connor, p. 103—108. London: Churchill 1960.

— A. Kokko and U. Söderholm: Starch electrophoretic separation of esterases in the adrenal medulla, adrenal cortex and the brain of the rat and the hamster. To be published.

Prof. O. Eränkö,
Department of Anatomy, University of Helsinki, Siltavuorenpenger 20
Helsinki, Finland

Reprint from Ann. Med. Exper. Fenn. Vol. 40. Fasc. 2, 1962

FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

EFFECT OF DENERVATION ON RESERPINE-INDUCED DEPLETION AND SUBSEQUENT RESTITUTION OF ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA OF THE RAT[1]

by

OLAVI ERÄNKÖ and VÄINÖ HOPSU

(Received for publication December 21, 1961)

The effect of reserpine on the adrenal medulla has been investigated in a large number of reports, ever since Carlsson and Hillarp (8) and Holzbauer and Vogt (21) independently observed that reserpine causes a loss of catecholamines from the medulla. However, the results obtained by different workers have been contradictory in many respects, to be discussed later in this paper. The present study was carried out to make clearer the significance of the splanchnic nerves in the response of the adrenal medulla to reserpine, a problem which has been the subject of sharply controversial observations and opinions.

MATERIAL AND METHODS

Experimental. — Adult male albino rats were used. It is necessary to note that these rats were not of the same strain as those used in the previous study (14), and they proved to have a different sensitivity towards reserpine.

The animals were given subcutaneous injections of reserpine. Four dosage levels were used: one group was given 0.5 mg per kg of body weight,

[1] This study has been supported by research grants from the Sigrid Jusélius Foundation, Helsinki, and from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service (A—1725).

another group 2 mg/kg, the third group 3 injections of 2 mg/kg in three consecutive days and the fourth group 3 injections of 4 mg/kg, the first two injections in two consecutive days, the third one first on the the fourth day because of the poor condition of the animals.

In the first series of experiments, the animals were unilaterally denervated about one week before the administration of reserpine by division of the left splanchnic nerves below the diaphragm. This procedure has earlier been observed to cause a total loss of acetylcholinesterase activity from the secretory nerve fibres of the adrenal medulla (16). The animals were killed 24 hours after the (last) injection of reserpine.

To study the effect of denervation on the resynthesis of the catecholamines in the adrenal medulla, the rats were first injected with 0.5 mg/kg, 2 mg/kg or 3 times 2 mg/kg of reserpine to cause an equal loss of catecholamines from both adrenals. These animals were unilaterally denervated about 24 hours after the (last) injection and killed 4—8 days thereafter.

Both unilaterally denervated and intact rats were employed as controls.

Estimation of Catecholamines. — Histochemical methods were used to assess the concentration of catecholamines in the adrenal medulla. The chromaffin reaction served for the demonstration of both catecholamines and the iodate reaction (19) for selective staining of the noradrenaline containing cells. The intensity of the histochemical reactions was quantitatively measured by microphotometry, which previously has been shown to furnish results which agree with chemical determinations (14, 15). For technical details the reader is referred to these two papers. However, the results have not been expressed directly in terms of optical density, as they were in these papers. In the present paper, 0.08 has been subtracted from the observed density value. This has been done to eliminate the effect of non-specific light loss due to light scattering even in entirely colourless sections, in which a density reading of about 0.08 was recorded. Thus, the values are given as units of density above the scattering density, which better reflect the intensity of the colour reactions and, accordingly, the concentration of catecholamines. To avoid unnecessary zeros, the results are expressed by multiplying the value density —0.08 by 100. The photometric measurements were carried out using randomly numbered, mixed slides without knowing to which group the gland belonged.

Student's t-test was used for calculating the probability P that the difference of the means is due to mere chance.

RESULTS

General Observations. — The results are presented in Tables 1 and 2. The intensity of the chromaffin reaction can be expected to be a good measure of the total concentration of both catecholamines, adrenaline and noradrenaline, in the adrenal medulla. The intensity of the iodate reaction in the noradrenaline cell islets and that in

the medullary 'background' outside the islets have been recorded separately. These variables are dependent on the medullary content of noradrenaline but the latter also depends on the relative amount of noradrenaline-containing cell islets in the medulla, a variable not measured in the present work. Although no such actual measurements were carried out for reasons discussed previously (14), visual examination showed that a decrease in the photometric density of the iodate positive islets was usually accompanied with a decrease in the relative amount of such islets, presumably because some islets had lost their noradrenaline stores before others and therefore apparently disappeared. On the other hand, a decrease in the intensity of the iodate reaction in the positive cell islets was never accompanied by a compensatory increase in the relative volume of islets issue which would make it possible that the mean medullary concentration of noradrenaline would remain the same or increase. Therefore, in judging the changes in the noradrenaline content of the adrenal medulla, it is fairly safe to assume that a decrease in the intensity of the iodate reaction in the noradrenaline cell islets is indicative of an at least equally large decrease in the mean medullary noradrenaline concentration, as has been observed previously by parallel histochemical and chemical determinations (14).

Denervation alone did not cause significant changes in the intensity of either reaction in the adrenal medulla of uninjected animals. Normal and unilaterally denervated controls were therefore pooled and regarded as one group, presented as the control group in Table 1.

Reserpine-Induced Depletion. — The effect of various doses of reserpine 24 hours after the (last) injection is shown in Table 1. The smallest dose employed, 0.5 mg/kg, caused but a slight loss of total catecholamines, as indicated by the mean intensity of the chromaffin reaction, and the decrease was statistically significant only in the right, intact adrenal. The intensity of the iodate reaction in the islets of the right adrenal medulla significantly decreased by about 80%, although the same variable did not significantly differ from normal in the denervated left gland. Accordingly, the left-right difference was highly significant. The intensity of the iodate reaction in the medullary background did not change significantly. These observations indicate, firstly, that denervation

TABLE 1

INTENSITY OF CHROMAFFIN AND IODATE REACTION IN THE ADRENAL MEDULLA OF RATS 24 HOURS AFTER (LAST) RESERPINE INJECTION

Intensity expressed as 100 × (optical density—0.08)
Left adrenals are denervated, right adrenals intact

Dose of Reserpine (mg/kg)	No.	Chromaffin Reaction				Iodate Reaction in Medullary Islets				Iodate Reaction in Medullary Background			
		Left		Right		Left		Right		Left		Right	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0 (Controls)	11	61.9	10.0	62.4	9.4	33.3	7.4	30.8	11.8	10.2	5.1	10.5	7.0
1 × 0.5	5	55.8	8.6	48.4	4.0	30.0	5.5	6.4	3.7	7.0	1.1	6.2	4.3
P_{CO}[1]		—		0.008		—		0.004		—		—	
P_{LR}[2]		—				0.000,2				—			
1 × 2.0	4	40.0	2.8	3.7	3.9	23.7	4.7	3.7	5.1	1.0	2.5	2.2	2.5
P_{CO}		0.001		0.000,1		0.04		0.001		0.005		0.004	
P_{LR}		0.000,1				0.001				—			
3 × 2.0	4	22.8	4.4	0.0	1.2	5.3	1.5	3.3	2.1	6.3	5.0	2.5	2.9
P_{CO}		0.000,1		0.000,1		0.000,1		0.000,8		0.000,1		0.000,1	
P_{LR}		0.000,1				—				—			
3 × 4.0	7	23.9	7.3	5.0	2.0	2.7	5.4	0.0	5.9	0.7	3.4	—0.4	1.5
P_{CO}		0.000,1		0.000,1		0.000,1		0.000,1		0.001		0.005	
P_{LR}		0.000,1				—				—			

[1]) Significance of difference of means, as compared with untreated controls.
[2]) Significance of left-right difference of means.

prevented the effect of this dose of reserpine and, secondly, that the loss of catecholamines from the intact adrenals was entirely or at least predominantly due to a decrease in the noradrenaline content of the medullary cell islets.

Larger doses, 2, 6 or 12 mg/kg of reserpine caused a significant decrease in the intensities of the chromaffin and iodate reaction in both the intact and the innervated adrenal. However, the left-

right difference in the intensity of the chromaffin reaction was highly significant in all of these experiments, indicating the preventive effect of denervation. In the innervated adrenals the decrease in the intensity of the chromaffin reaction was 92—100%, which shows that reserpine had caused in them a nearby total loss of both adrenaline and noradrenaline.

A single dose of 2 mg/kg was capable of causing a significant but relatively slight (about 25%) loss of noradrenaline also from the islets of the denervated adrenals, while the mean density of the iodate reaction in the islets of the innervated gland had decreased by about 90%. The left-right difference was statistically significant. It is of interest that the mean 'background' density of even the non-denervated adrenals at the same time decreased significantly.

Three times 2 mg/kg or 3 times 4 mg/kg decreased the optical density of the iodate reaction in the medullary cell islets and in the background to very low values both in the intact and the denervated adrenals, and the left-right differences were not statistically significant.

To sum up, reserpine caused a loss of catecholamines dependent on dosage and innervation in such a way that small doses were followed by a predominant depletion of noradrenaline, which was dependent on intact innervation, while progressively larger doses decreased also the adrenaline content and caused a loss of catecholamines even from the denervated adrenals.

Recovery after Depletion. — Table 2 illustrates the recovery of the catecholamines after depletion at 3 different dosage levels. In the table are repeated the corresponding values of the right (intact) adrenal 24 hours after the (last) reserpine injection. These values serve as stimulation controls. Because, in the recovery experiments, the animals were first injected with reserpine and denervated 24 hours thereafter, their both adrenals were depleted to about the same level as the intact adrenal of the animals first denervated and then injected with reserpine. The left-right comparison thus gives an idea of the effect of innervation on restitution in equally depleted adrenals.

Five days after a single injection of 0.5 mg/kg, the intensity of the chromaffin reaction did not differ significantly from that of uninjected controls, either in the denervated or the intact adrenals.

TABLE 2

INTENSITIES OF CHROMAFFIN AND IODATE REACTIONS IN THE ADRENAL MEDULLA OF RATS INJECTED WITH RESERPINE, UNILATERALLY DENERVATED 1 DAY AFTER THE (LAST) INJECTION AND ALLOWED TO RECOVER THEREAFTER

Intensity expressed as 100 × (optical density—0.08)
Left adrenals are denervated, right ones intact

Dose (mg/kg)	Killed Days after Injection)	No	Chromaffin Reaction		Iodate Reaction in Medullary Islets		Iodate Reaction in Medullary Background	
			Left	Right	Left	Right	Left	Right
			Mean SD	Mean SD	Mean SD	Mean SD	Mean SD	Mean SD
0.5	1	5		48.4 4.0		6.4 3.7		6.2 4.3
	5	3	60.3 6.0	54.0 2.7	30.3 2.1	20.0 1.0	14.0 1.0	10.3 1.6
P_{CO}[1]			—	—	—	—	—	—
P_{ST}[2]			0.015	—	0.000,1	0.001	0.03	—
P_{LR}[3]			—		0.002		0.03	
2.0	1	4		3.7 3.9		3.7 5.1		2.2 2.5
	5	5	52.4 7.7	48.5 4.0	13.8 2.7	10.2 5.0	9.7 1.0	7.6 5.1
P_{CO}			—	0.02	0.000,3	0.003	—	—
P_{ST}			0.000,1	0.000,1	—	—	0.002	—
P_{LR}			—		—		—	
	9	5	56.4 6.4	53.4 9.7	15.2 4.8	6.0 3.4	7.6 3.2	5.0 4.1
P_{CO}			—	—	0.000,2	0.002	—	—
P_{ST}			0.000,1	0.000,1	0.01	—	0.04	—
P_{LR}			—		0.02		—	
3×2.0	1	4		0.0 1.2		3.3 2.1		2.5 2.9
	8	5	42.8 8.0	38.8 7.3	21.4 4.4	16.4 5.6	13.0 3.5	14.4 4.4
P_{CO}			0.002	0.000,3	0.005	0.03	—	—
P_{ST}			0.000,1	0.000,1	0.000,1	0.002	0.001	0.002
P_{LR}			—		—		—	

[1] Significance of difference of means, as compared with untreated controls (Table 1).

[2] Significance of difference of means as compared with stimulation controls killed 24 hours after (last) injection.

[3] Significance of left-right difference of means.

The powerful, about 80%, decrease in the intensity of the iodate reaction in the medullary cell islets due to this dose 24 hours after the injection recovered to a great extent but the magnitude of the recovery was smaller in the right, intact adrenal than in the left, denervated gland, and the left-right difference was statistically significant. A similar, though less significant left-right difference was observed in the intensity of the iodate reaction in the medullary background.

Both 5 and 9 days after an injection of 2 mg/kg of reserpine, the intensity of the chromaffin reaction had returned to 78—90% of the normal control value, the difference from the value of the 24 hours stimulation control being highly significant. At the same time, the noradrenaline content of the medullary cell islets, as indicated by the iodate reaction, increased from the low 24 hours stimulation value (12% of normal) but reached only a level of 18—48% of normal, the difference from the normal control value being in all cases significant. The mean intensity of the iodate reaction in the background also increased and reached a level not significantly different from that of normal controls.

Eight days after 3 times 2 mg/kg of reserpine, the intensity of the chromaffin reaction significantly increased after the total depletion observed 24 hours after similar treatment, but the means of both denervated and intact adrenals significantly differred also from the normal control value. The relative recovery in the intensity of the iodate reaction in the islets was of the same magnitude order as that in the chromaffin reaction, the mean values of the denervated and intact glands significantly differring from those of both stimulation controls and untreated controls. The background iodate reaction was not significantly different from that of normal controls.

Significant left-right differences were in the recovery experiments recorded only in three instances, *i.e.* in the iodate reaction of the medullary islets and of the background 5 days after 0.5 mg/kg, as well as in the iodate reaction of the islets 9 days after 2 mg/kg. However, with the single exception of the intensity of the iodate reaction in the medullary background 8 days after 3 times 2 mg/kg, which in both the left and the right adrenal had already reached a value not significantly different from that of normal, the mean intensities of both the chromaffin reaction and the iodate reaction were always higher in the denervated than in the innervated glands.

Under the present experimental conditions, then, significant recovery was recorded after depletion induced by 0.5, 2 or 6 mg/kg of reserpine. Restitution of noradrenaline was slower than that of adrenaline, and denervation had a favourable effect on the rate of restitution of the catecholamines.

DISCUSSION

In the present study, a dose of 2 mg/kg of reserpine was sufficient to cause an almost complete depletion of both adrenaline and noradrenaline from intact adrenals. The same dose was also used by Camanni, Losana, Molinatti and Olivetti (7), with similar results. On the other hand, in a previous study from our laboratory (13), in which rats of another strain were employed, a larger dose, 3 mg/kg, resulted in a loss of only about 20% or less of the catecholamine content, and Kroneberg and Schümann (23) found, also in rats, a depletion of 25—68% with a still larger dose of 10 mg/kg. Comparison of published data on the catecholamine response of rat's adrenals to reserpine further shows that there are great variations in the sensitivity of different rats, presumably conditioned by the season, the strain, the diet and other environmental factors (4, 6, 7, 9, 11, 13, 14, 18, 23, 25, 26).

In spite of the great variation in the sensitivity of rats towards reserpine many workers have observed that noradrenaline is preferentially, often selectively, lost from the adrenal medulla, although the adrenaline stores can also be depleted by increasing the dosage (6, 7, 13, 14, 23, 25). Stjärne and Schapiro (28), moreover, found that reserpine much increases the secretion of noradrenaline into the adrenal vein but has less effect on adrenaline secretion of cat's adrenals. The present work, in which the loss of catecholamines caused by 0.5 mg/kg of reserpine was mainly limited to noradrenaline, is in agreement with these observations. On the other hand, Callingham and Mann (4) and Coupland (9) observed an equal relative loss of adrenaline and noradrenaline even when the magnitude of total depletion was 50% or less. It is difficult to explain this discrepancy, but it is not due to differences in the sensitivity of the rats used, since the dosage employed by these workers was within the range covered in the experiments by others (see above) in which preferential depletion of noradrenaline was observed.

Reports concerning the significance of nervous transmission in the catecholamine depletion by reserpine are variable. Some workers have observed that the effect of reserpine is not at all affected by splanchnic denervation (5, 26), while others have found that denervation either diminishes or prevents the reserpine-induced loss of catecholamines (7, 13, 18, 21, 23, 24, 27).

The present work indicates that the dosage of reserpine is of essential importance in this respect: the effect of small doses is entirely or essentially prevented by denervation, while large doses are capable of depleting also the denervated adrenals. This observation partly explains the variations between previous studies and is in conformity with the results obtained by Greenberg, Jeffay and Toman (17), who studied the effect of deserpidine on intact and denervated rat's adrenals. It fits also in with Coupland's (10) observation, made using rats, that injection of reserpine in a dose sufficient to cause an almost complete loss of adrenaline and noradrenaline from intact adrenals is followed by a distinct but smaller loss of amines from autografts of adrenal medulla in the anterior chamber of the eye. On the other hand, our observations are even in this respect at variance with those by Mirkin (26) and Callingham and Mann (5), and again the discrepancy cannot be explained by the dosage level.

Reserpine exerts its action on the adrenal medulla through several complicated mechanisms. It can cause an increased output of catecholamines from the adrenal medulla (25, 28). Even after complete denervation a certain, though decidedly smaller, stimulating effect on the output remains (29). Further, reserpine seriously affects the balance of catecholamines between the storage granules and the soluble cytoplasmic pool of the secretory cells of the adrenal medulla by preventing the otherwise rapid binding of catecholamines in the granules (1). On the other hand, splanchnic stimulation, which according to the observations discussed above can be expected to play an important role in the effect of reserpine on the adrenal medulla, has been reported to increase not only the output but also the formation of catecholamines in the adrenal medulla (2, 3, 20 22). Indeed, Stjärne and Schapiro (28) have obtained results suggesting an increased rate of synthesis of noradrenaline 2—3 hours after the administration of reserpine. The same authors pointed out, however, that in the long run synthesis does not keep

pace with secretion. Leduc (25) has recently presented indirect evidence of an inhibitory effect of reserpine on the synthesis of catecholamines in the adrenal medulla.

Since the balance of output and synthesis, which determines the catecholamine concentration of the adrenal medulla, can be influenced through several simultaneous but different actions of reserpine, it is understandable that slight differences in the experimental conditions may lead to different types of depletion patterns. This probably further explains the variability of results obtained by different investigators.

In the present study, restitution of catecholamines after depletion was more rapid in denervated adrenals than in intact adrenals. To our knowledge such a phenomenon has not earlier been observed. Callingham and Mann (5) found no difference in the replacement of adrenaline or noradrenaline between innervated and denervated adrenals of the rat after reserpine-induced depletion, neither did Greenberg, Jeffay and Toman (17) in rats depleted by deserpidine. According to Coupland (12), autografts of rat's adrenal medulla, first depleted of catecholamines by reserpine and then inserted in the anterior chamber of the eye, are capable of replacing the adrenaline and noradrenaline content at about the same rate as intact adrenals. Kroneberg and Schümann (24), in an experiment similar to ours but performed on rabbits, observed that the rate of recovery of adrenaline was slower in the denervated adrenal than that in the intact one.

In all of these studies denervated adrenomedullary cells were capable of recovering their normal catecholamine stores after depletion at a rate not much different from that of normally innervated cells. Although nervous stimulation has been reported to promote both output and synthesis of catecholamines (2, 3, 20, 22, 28), it is clear that nervous stimulation usually results in an output equal or higher than resynthesis, the net effect being that the catecholamine concentration remains the same or decreases.

If nervous stimulation affects in the same way adrenals with ample stores of catecholamines as adrenals depleted of their stores, which has as yet not been investigated, one would expect that the rate of resynthesis of catecholamines in depleted and subsequently denervated adrenals would be equal or higher than that in depleted intact adrenals, as indeed has been observed in most studies (5, 12,

17, the present work). On the other hand, a difference in the rate of restitution between intact and denervated adrenals in favour of the former (24) necessitates either that nervous stimulation can directly increase the resynthesis rate, of which evidence is not available, or that denervation somehow interferes with the synthesis. Obviously further studies are necessary to clarify these problems.

SUMMARY

Concentrations of adrenaline and noradrenaline were estimated microphotometrically in sections in which the catecholamines had been demonstrated histochemically using the chromaffin reaction and the iodate reaction. Small doses of reserpine caused a selective loss of noradrenaline, which was prevented by splanchnic denervation. Large doses induced a loss of both amines both in intact and in denervated adrenals, although the loss was always smaller in the denervated gland. Restitution of catecholamine concentrations was more rapid in the adrenal medulla of glands denervated after depletion than that in intact adrenals.

REFERENCES

1. Bertler, Å., Hillarp, N. Å., and Rosengren, E.: Experientia (Basel) 1960:*16:*419.
2. Bygdeman, S., and v. Euler, U. S.: Acta physiol. scand. 1958:*44:*375.
3. Bygdeman, S., v. Euler, U. S., and Hökfelt, B.: Acta physiol. scand. 1960:*49:*21.
4. Callingham, B. A., and Mann, M.: Nature (Lond.) 1958:*181:*423.
5. Callingham, B. A., and Mann, M.: Nature (Lond.) 1958:*182:*1020.
6. Camanni, F., Losana, O., and Molinatti, G. M.: Experientia (Basel) 1958:*14:*199.
7. Camanni, F., Losana, O., Molinatti, G. M., and Olivetti, M.: Arch. int. Pharmacodyn. 1960:*123:*430.
8. Carlsson, A., and Hillarp, N. Å.: Kungl. fysiogr. Sällsk. Lund Förh. 1956:*26:*8.
9. Coupland, R. E.: Nature (Lond.) 1958:*181:*930.
10. Coupland, R. E.: J. Endocr. 1958:*17:*191.
11. Coupland, R. E.: J. Endocr. 1959:*18:*154.
12. Coupland, R. E.: J. Endocr. 1959:*18:*162.
13. Eränkö, O., and Hopsu, V.: Endocrinology 1958:*62:*15.
14. Eränkö, O., and Hopsu, V.: Acta physiol. scand. 1961:*51:*239.
15. Eränkö, O., and Räisänen, L.: J. Histochem. Cytochem. 1961:*9:*54.
16. Eränkö, O., Hopsu, V., and Räisänen, L.: J. Neurochem. 1959:*4:*264.

17. GREENBERG, R., JEFFAY, A. I., and TOMAN, J. E. P.: J. Pharmacol. exp. Ther. 1960:*130:*119.
18. HILLARP, N. Å.: Nature (Lond.) 1960:*187:*1032.
19. HILLARP, N. Å., and HÖKFELT, B.: J. Histochem. Cytochem. 1955:*3:*1.
20. HOLLAND, W. C., and SCHÜMANN, H. J.: Brit. J. Pharmacol. 1956:*11:* 449.
21. HOLZBAUER, M., and VOGT, M.: J. Neurochem. 1956:*1:*8.
22. HÖKFELT, B., and MCLEAN, J.: Acta physiol. scand. 1950:*21:*258.
23. KRONEBERG, G., and SCHÜMANN, H. J.: Arch. exp. Path. Parmak. 1957:*231:*349.
24. KRONEBERG, G., and SCHÜMANN, H. J.: Experientia (Basel) 1959:*15:* 234.
25. LEDUC, J.: Acta physiol. scand. 1961:*53:*Suppl. 183.
26. MIRKIN, B. L.: Nature (Lond.) 1958:*182:*113.
27. MUSCHOLL, E., and VOGT, M.: J. Physiol. (Lond.) 1958:*141:*132.
28. STJÄRNE, L., and SCHAPIRO, S.: Nature (Lond.) 1958:*182:*1450.
29. STJÄRNE, L., and SCHAPIRO, S.: Nature (Lond.) 1959:*184:*2023.

Acta physiol. scand. 1963. 58. 285—286

From the Department of Anatomy, University of Helsinki,
Siltavuorenpenger, Helsinki, Finland

Histochemical Demonstration of Fluorogenic Amines in the Cytoplasm of Sympathetic Ganglion Cells of the Rat

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

Sympathetic ganglia are known to contain catecholamines, predominantly noradrenaline (EULER 1947, VOGT 1954). However, little is known of the distribution of these amines within the ganglion. In a study mainly dealing with the peripheral sympathetic structures, FALCK (1962) briefly stated that a varying number of ganglion cells in adrenergic ganglia show a positive histochemical reaction for noradrenaline in the cytoplasm and in the larger processes. We are not aware of any other study in this subject.

Formalin-induced fluorescence has long been used for histochemical demonstration of noradrenaline-containing chromaffin cells (ERÄNKÖ 1955). Only recently it was observed independently in 3 laboratories that dry formaldehyde vapour converts several amines into strongly fluorescent compounds, whose distribution in frozen-dried sections can be studied by fluorescence microscopy (ERÄNKÖ 1961, 1963, FALCK and TORP 1961, FALCK 1962, LAGUNOFF, PHILLIPS and BENDITT 1961). The present report deals with the superior cervical ganglion of the rat, in which noradrenaline can be expected to be the main fluorogenic amine. Fresh ganglia were frozen and dried at — 40° C. Freeze-dried tissue was exposed to dry formaldehyde vapour before or after embedding in paraffin wax and sectioning (for details see ERÄNKÖ 1963).

A green fluorescence was observed in the cytoplasm of all ganglion cells and in nerve fibres between them (Fig. 1). The fluorescence intensity varied from one cell to another, ranging from weak to strong, through intermediate stages. A diffuse cytoplasmic fluorescence was seen in all nerve cells but, in addition to it, brilliantly fluorescent small granules were observed in the cytoplasm of many cells and in the fibres between the cells. In cells whose axons were visible in the section the view suggested migration of the granules from the cytoplasm to the axon (Fig. 2). Especially near bundles of nerve fibres, occasional small cells exhibited an extremely bright yellow fluorescence. These cells were very few, and apparently they were not nerve cells. Ganglia taken from rats whose catecholamine stores had been depleted with large doses of reserpine were essentially non-fluorescent.

The observations described suggest (1) that sympathetic ganglion cells manufacture and store catecholamines in the cytoplasm (2) that the catecholamine content greatly varies in individual cells, (3) that a part of the cytoplasmic

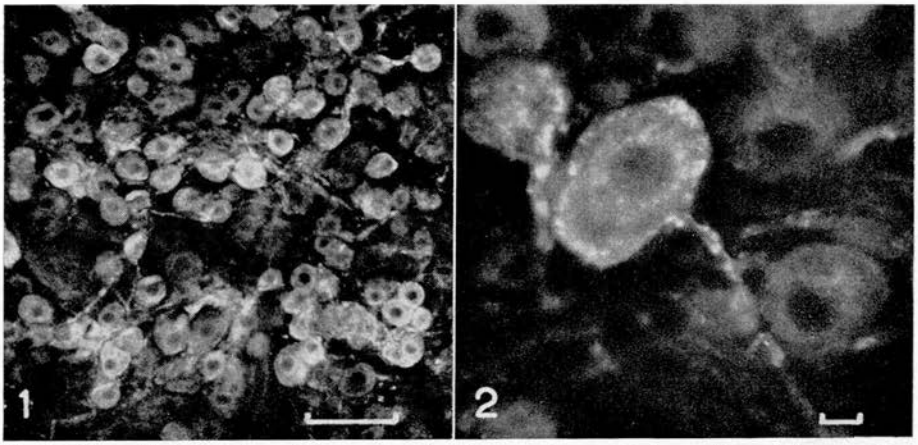


Fig. 1. Formaldehyde-induced fluorescence in the superior cervical ganglion of the rat. Note great variations in the fluorescence intensity of individual cells. Siemens lamp HBO 200, Schott filters BG 12 and OG 1. Scale mark 100 μ.
Fig. 2. Cells from the same specimen. Brightly fluorescent granules are visible in the diffusely fluorescent cytoplasm and along the axon originating from it. Scale mark 10 μ.

catecholamine is in a soluble form, while another part is concentrated in granules, and (4) that these granules migrate from the cytoplasm to the axon.

These tentative conclusions, which should be tested by other methods such as electron microscopy and analysis of cytoplasmic granule fractions, fit well in with the recent observations which indicate that noradrenaline is partly soluble, partly bound in granules in sympathetic nerves and in adrenergic nerve terminals (EULER and HILLARP 1956, FALCK 1962).

This study has been supported by grants from the Sigrid Jusélius Foundation, Helsinki, and from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

References

ERÄNKÖ, O., Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. *Acta endocr.* (Kbh.) 1955. *18.* 174—179.

ERÄNKÖ, O., Personal communication to Dr. FALCK, acknowledged in FALCK and TORP (1961).

ERÄNKÖ, O., Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *To be publ.* 1963.

EULER, U. S. v., A specific sympathomimetic ergon in adrenergic nerve fibres (sympathin) and its relations to adrenaline and noradrenaline. *Acta physiol. scand.* 1947. *12.* 73—97.

EULER, U. S. v. and N.-Å. HILLARP, Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Nature* (*Lond.*) 1956. *177.* 44—45.

FALCK, B., Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.* 1962. *56.* Suppl. 197. pp. 25.

FALCK, B. and A. TORP, A fluorescence method for histochemical demonstration of noradrenalin in the adrenal medulla. *Med. exp.* 1961. *5.* 429—432.

LAGUNOFF, D., M. PHILLIPS and E. V. BENDITT, The histochemical demonstration of histamine in mast cells. *J. Histochem. Cytochem.* 1961. *9.* 534—541.

VOGT, M., The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs. *J. Physiol.* (*Lond.*) 1954. *123.* 451—481.

KUNGL. BOKTR. STHLM 1963

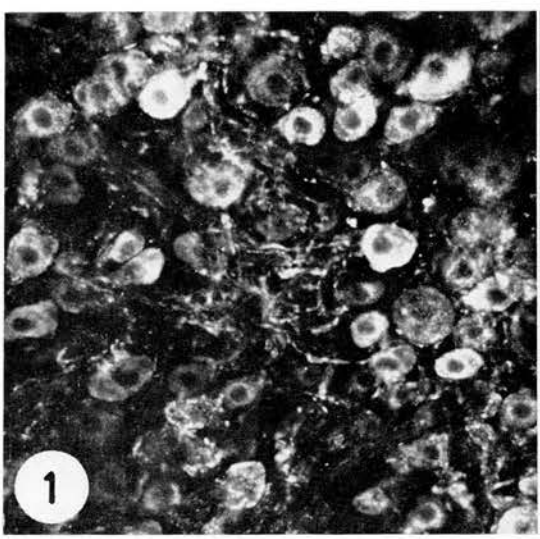
Reprinted from THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 12, No. 6 June, 1964

HISTOCHEMICAL DEMONSTRATION OF CATECHOLAMINES BY FLUORESCENCE INDUCED BY FORMALDEHYDE VAPOUR

Fluorescence induced by formaldehyde was first described in the adrenal medulla (Eränkö, *Acta anat.* **16:** Supp. 17, 1952), in which the noradrenaline-containing cells were found to be responsible for the fluorescence (Eränkö, *Acta Endocrinol.* **18:** 174, 1955). It was later observed that dry formaldehyde vapour converts several monoamines into intensely fluorescent compounds in frozen dried tissues (Lagunoff, Phillips and Benditt, *J. Histochem.* **9:** 534, 1961; Falck and Torp, *Med. Exp.* **5:** 429, 1961; Eränkö, personal communication acknowledged by Falck and Torp, *ibid.*) Based on this principle, a method was published by Falck (*Acta Physiol. Scand.* **56:** Supp. 197, 1962) which was suitable for accurate histochemical demonstration of monoamines. Frozen dried tissues were exposed for 2 hours to dry formaldehyde vapour at 80°C and embedded in paraffin wax. Since such treatment inactivates the enzymes, formaldehyde treatment of dried cryostat sections was recently proposed by Hamberger and Norberg (*J. Histochem.* **12:** 48, 1964) for the study of monoamines, whose distribution could then be compared with that of an enzyme histochemically demonstrated in a neighbouring section.

A method is reported in the present paper which renders it possible to demonstrate consecutively in the same section, first, the fluorescence due to monoamines and, subsequently, a histochemical enzyme reaction. The diffusion artifacts which limit the value of the cryostat section technique (Hamberger and Norberg *loc cit.*) are completely avoided in this method.

Small pieces of tissue were quickly frozen using metal disc forceps precooled in liquid air (Eränkö, *Acta Anat.* **22:** 331, 1954). The pieces were dried in a short-path freeze-drying apparatus embodying a diffusion pump and a liquid air trap. The dry pieces were then treated with formaldehyde vapour from paraformaldehyde, varying the temperature from 40 through 80°C and the time of exposure from 15 min through several hours. Different amines were thus found to be converted into fluorescent compounds at different rates, which made it possible to discriminate, e.g., between adrenaline and noradrenaline. Exposure for 1 hour at 50°C was sufficient to make nervous tissue fluorescent enough for microscopic localization of amines but did not yet inactivate acetylcholinesterase, non-specific cholinesterase, non-specific esterases, lactic dehydrogenase or tetrazolium reductases, which can subsequently be histochemically demonstrated. Exposure to ultraviolet light



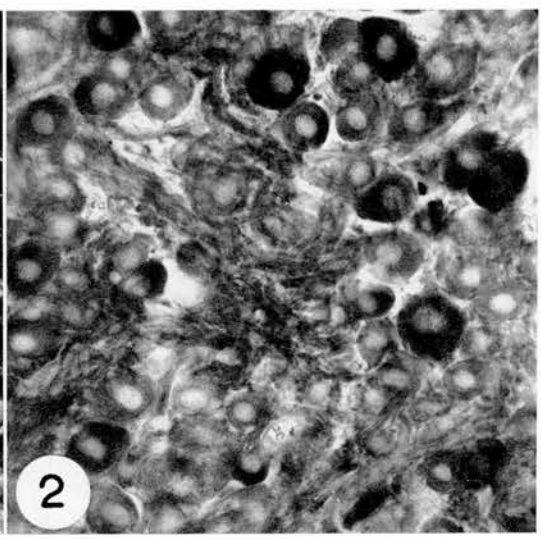


FIG. 1. Fluorescence photomicrograph of rat's superior cervical ganglion. Exposure to formaldehyde vapour for 1 hour at 50°C. Note the variation in the fluorescence intensity of individual cells.
FIG. 2. The same section after demonstration of esterases with α-naphthyl acetate and Fast Blue RR

must be limited to minimum, because it gradually inactivates the enzymes.

Fig. 1 shows the fluorescence due to noradrenaline in the superior cervical ganglion of the rat. Fig. 2 is of the same section after demonstration of esterases with α-naphthyl acetate. In a pair of photomicrographs such as this, the amine concentration can be directly compared with the enzyme activity of exactly the same cell or even a smaller tissue component. A study in which acetylcholinesterase activity has been compared with amine content is published elsewhere (Eränkö and Härkönen, *Acta Physiol. Scand.*, in press).

Alternatively, frozen dried tissues were embedded in paraffin wax and sections deparaffinized with benzene were first thereafter subjected to formaldehyde vapour. This makes it more convenient to study the time-temperature characteristics of the amines involved. Enzymes such as amine oxidase, which are inactivated even by a mild formaldehyde treatment, can be studied in neighbouring sections not exposed to formaldehyde.

The two methods presented are capable of accurate localization of both fluorogenic amines and histochemically demonstrable enzymes. With an efficient equipment, the freeze-drying process can without sacrifice in quality be reduced to 2 or 3 days, which is not an unduly long period in view of the improved localization, as compared with the cryostat method. However, the latter has obvious advantages as an exploratory method, and it is the only possible technique by which the distribution of amines can be compared with that of highly sensitive enzymes, e.g. succinic dehydrogenase.

March 26, 1964

Olavi Eränkö
Department of Anatomy
University of Helsinki
Siltavuorenpenger
Helsinki, Finland

Acta physiol. scand. 1964. 61. 299—300

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Noradrenaline and Acetylcholinesterase in Sympathetic Ganglion Cells of the Rat

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

In a previous histochemical study, it was shown that the noradrenaline (NA) concentration of individual nerve cells of the superior cervical ganglion of the rat ranges from low to high, through intermediate stages (Eränkö and Härkönen 1963). In the present report, the distribution of NA is compared with that of acetylcholinesterase (AChE) activity.

A method was employed which allows demonstration of NA and AChE in the same section (Eränkö 1964). Frozen dried superior cervical ganglia of the rat were exposed to dry formaldehyde vapour at 50—55° C for 1 hour, which makes NA fluorescent but does not destroy AChE. After fluorescence photomicrography, AChE activity was demonstrated with Gomori's (1952) method, using acetylthiocholine as a substrate and 10^{-6} M tetra-isopropylpyrophosphoramide to inhibit non-specific cholinesterase.

Fig. 1 shows formaldehyde-induced fluorescence due to NA. Fig. 2 is of the same section after demonstration of AChE. Examination of such pairs of photomicrographs proved that weak, moderate or strong fluorescence may be associated with weak, moderate or strong AChE activity. Many strongly fluorescent cells exhibited a marked AChE reaction.

If it is accepted that nerve cells with much NA are adrenergic and those with an intense AChE activity cholinergic (see Koelle 1962), our results suggest that some sympathetic ganglion cells may be both adrenergic and cholinergic at the same time. This is of special interest in view of pharmacological observations apparently indicating that stimulation of a cholinergic sympathetic fiber causes a liberation of acetylcholine, which in turn may exert its effect by liberating NA from the same fiber or an adjacent one (Burn and Rand 1962). Our preliminary studies have indeed suggested that some nerve fibers of the rat iris may contain both NA and AChE.

Hamberger, Norberg and Sjöqvist (1963) compared the distributions of NA and AChE in neighbouring sections of cat's sympathetic ganglia. In contrast to our observations, they never found monoamines and significant levels of AChE in the same neuron. The discrepancy between their and our observations may well be due to species differences (see Burn and Rand 1962, Koelle 1962).

This study has been supported by grants from the Sigrid Jusélius Foundation and from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

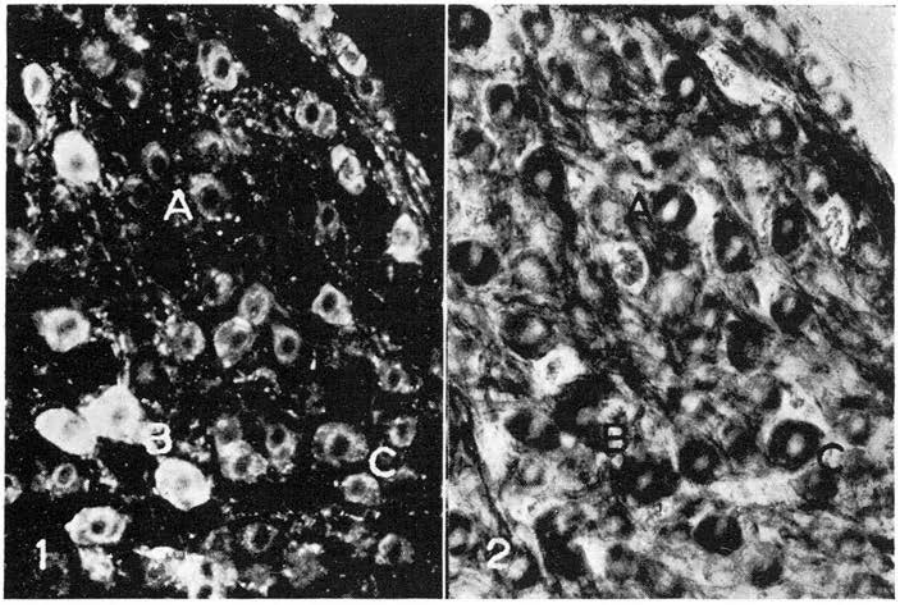


Fig. 1. Formaldehyde-induced fluorescence in the superior cervical ganglion of the rat, showing the distribution of noradrenaline.

Fig. 2. The same section after demonstration of acetylcholinesterase activity. Three corresponding sites in both figures are indicated with letters, to the left of which are: A, two weakly fluorescent cells with a weak acetylcholinesterase activity; B, two strongly fluorescent cells with a strong acetylcholinesterase activity; C, two moderately fluorescent cells with a strong acetylcholinesterase activity.

References

BURN, J. H. and M. J. RAND, A new interpretation of the adrenergic nerve fiber. In *Advances in Pharmacology* (Ed. S. GARATTINI and P. A. SHORE) Vol. *1*. 1—30. Academic Press, New York and London. 1962.

ERÄNKÖ, O., Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.* 1964. In press.

ERÄNKÖ, O. and M. HÄRKÖNEN, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58*. 285—286.

GOMORI, G., *Microscopic Histochemistry, Principles and Practice*. The University of Chicago Press, Chicago. 1952.

HAMBERGER, B., K.-A. NORBERG and F. SJÖQVIST, Cellular localization of monoamines in sympathetic ganglia of the cat. *Life Sciences* 1963. *9*. 659—661.

KOELLE, G. B. A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. *J. Pharm. Pharmacol.* 1962. *14*. 65—90.

KUNGL. BOKTR. STHLM 1964

Reprinted from THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 12, No. 8 September, 1964

HISTOCHEMICAL AND STARCH GEL ELECTROPHORETIC CHARACTERIZATION OF DESMO- AND LYO-ESTERASES IN THE SYMPATHETIC AND SPINAL GANGLIA OF THE RAT*

OLAVI ERÄNKÖ,† MATTI HÄRKÖNEN, AULIKKI KOKKO AND LIISA RÄISÄNEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received for publication March 4, 1964

SYMPHORESIS

Esterases of the sympathetic and spinal ganglia of the rat were studied in sections and in starch slabs after electrophoresis, employing α-naphthyl acetate, naphthol AS-D acetate, 4-chloro-5-bromoindoxyl acetate and acetylthiocholine as substrates, and 1:5-bis-(4-allyl dimethylammoniumphenyl)pentan-3-one diiodide (284C51), tetra-isopropylpyrophosphoramide (iso-OMPA), eserine, and diethyl-*p*-nitrophenyl phosphate (E600) as inhibitors. Extracts of lyo-esterases were prepared by brief treatment of fresh-frozen sections with water, extracts of desmo-esterases by repeated freezing, thawing and homogenization of washed frozen sections. These extracts were subjected to starch gel electrophoresis. The effect of extraction with water on histochemical reactions in sections was studied in parallel.

Only desmo-esterases were observed to be responsible for the histochemical reactions in fresh sections. Lyo-zymograms exhibited band patterns different from those in desmo-zymograms but essentially similar to those in zymograms prepared from homogenates. Relative intensities of different types of esterase activity demonstrable in sections were similar to those in desmo-zymograms and, accordingly, different from those in homogenate or lyo-zymograms.

After formalin fixation, positive histochemical reactions were observed in structures which were negative in fresh sections, apparently because formalin immobilized lyo-esterases. At the same time, formalin inactivated some desmo-esterases. Thus, different enzyme spectra were observed in fresh and in fixed sections. By comparing reactions obtained with several substrate-inhibitor combinations in fresh and fixed sections, as well as in lyo- and desmo-zymograms, it was possible to correlate a histochemical reaction in a section with a specific area in the zymogram.

Since the total esterase activity of neural tissue is thus clearly due to several enzymes with differences in distribution, solubility and electrophoretic mobility, as well as in behaviour towards different substrates, inhibitors and fixing agents, a sufficient variety of methods discriminating between the different esterases is necessary to further investigate their nature and functional significance.

* The material presented here was presented originally at the Symposium on the Function of Esterases in Animals, University of Recife, Recife, Pernambuco, Brazil, September 8–12, 1963.

† Supported by a research grant from the Sigrid Jusélius Foundation, Helsinki.

INTRODUCTION

It has been demonstrated that histochemical esterase reactions show different localizations in tissue sections according to the substrates or inhibitors employed (5, 7, 9, 11, 14, 21, 22). This suggests the presence of several substrate-specific esterases, and starch gel electrophoretic studies have also indicated such multiplicity of esterases (1, 2, 3, 6, 8, 9, 16, 18). Esterases have furthermore been demonstrated to be partly readily soluble (lyo-esterases) and partly poorly soluble or firmly attached to tissue (desmo-esterases) (1, 3, 8, 13, 19). Although observations have been reported which suggest that esterases of the lyo-fraction, which can readily be studied by electrophoresis, may possess enzymic properties different from those of esterases of the desmo-fraction, mainly responsible for histochemical reactions in sections (1, 8, 9, 13, 19), little is known of such differences. Therefore, it appeared desirable to investigate the properties of lyo- and desmo-esterases in tissue sections and in zymograms, using suitable substrates and inhibitors to characterize the different types of esterase activity.

MATERIAL AND METHODS

1. Preparation of sections: Albino rats, descendants of the Sprague-Dawley strain, were

killed by cutting the neck under ether anaesthesia. Superior cervical ganglia and several spinal ganglia were thereafter removed.

Fresh frozen sections cut at 5–20 μ with a cryostat microtome were allowed to thaw and dry on coverslips. Alternatively, the ganglia were first fixed in a solution containing 1 volume of 35% HCHO, 6 volumes of 2% $CaCl_2$ and 3 volumes of H_2O. The fixation was carried out for 3 hours at room temperature. Frozen sections of fixed ganglia were treated free-floating with the histochemical procedures.

2. Starch gel electrophoresis: The technique was essentially that described by Markert and Hunter (18). Tissues homogenized in an equal volume of distilled water were frozen and thawed 6 times and centrifuged for 30 min at 25,000 $\times$ g. About 10 μl of the clear supernatant was applied to a filter paper strip, and this was inserted in a slot made in the starch slab.

Extracts of lyo-enzymes were prepared by covering dry fresh sections with distilled water, which was removed after 1 min. The sections were then immersed in water for 30 min to remove the rest of soluble enzymes. They were then dried, transferred into a homogenizer with water, and homogenized, frozen and thawed 6 times to liberate the desmo-enzymes. Both the lyo- and the desmo-extract were then centrifuged as the homogenate, and the supernatants were used.

A voltage gradient of 6 V/cm for 2.5–3 hours was employed for electrophoresis. The 9 mm thick starch slabs were then sliced into three 3 mm thick slices, which were developed with different substrate-inhibitor combinations.

3. Histochemical reactions: The following substrates were employed: α-naphthyl acetate (20), naphthol AS-D acetate (12), acetylthiocholine (10, 17) and 4-chloro-5-bromoindoxyl acetate (15). The techniques were essentially as given in the references, with some modifications. Thus, only fast blue RR salt was used as a coupling agent with the naphtholic substrates. For sections, its concentration was increased to 5 or 7.5 mg/ml when α-naphthyl acetate was used as a substrate to improve the localization (1). For starch gels, a concentration of 1 mg/ml was used. With 4-chloro-5-bromoindoxyl acetate, the originally prescribed 5 mM ferri-ferrocyanide mixture was employed for sections, while a 0.5 mM mixture (3) was used for starch slabs, with clearly improved sensitivity.

4. Esterase inhibitors: Eserine in a concentration of 10^{-5} M was used to inhibit cholinesterases (ChE). To discriminate between acetylcholinesterase (AChE) and non-specific cholinesterase (ns.ChE), 10^{-6} M 1:5-bis-(4-allyl dimethylammoniumphenyl)pentan-3-one diiodide (284C51) was employed as a specific inhibitor of AChE and 10^{-6} M tetra-isopropylpyrophosphoramide (iso-OMPA) as a specific inhibitor of ns.ChE (4, 5, 14, 21). Diethyl-p-nitrophenyl phosphate (E600) in a concentration of 10^{-5} M served for discriminating between two types of eserine resistant esterase activity (7, 8, 21). In the following description, activity resistant to eserine but sensitive to E600 is referred to simply as non-specific esterase (ns.E) activity, and activity resistant to both of these inhibitors as E600 resistant non-specific esterase (E-r.ns.E) activity, even if it is realized that this type of activity may be due to peptidases such as cathepsin (21).

The inhibitors were contained in the above concentrations not only in the substrate mixtures but also in pre-incubation mixtures similar to the former save for the substrate. Both slabs and sections were pre-incubated with the inhibitor for 30 min before immersion in the substrate mixture.

RESULTS

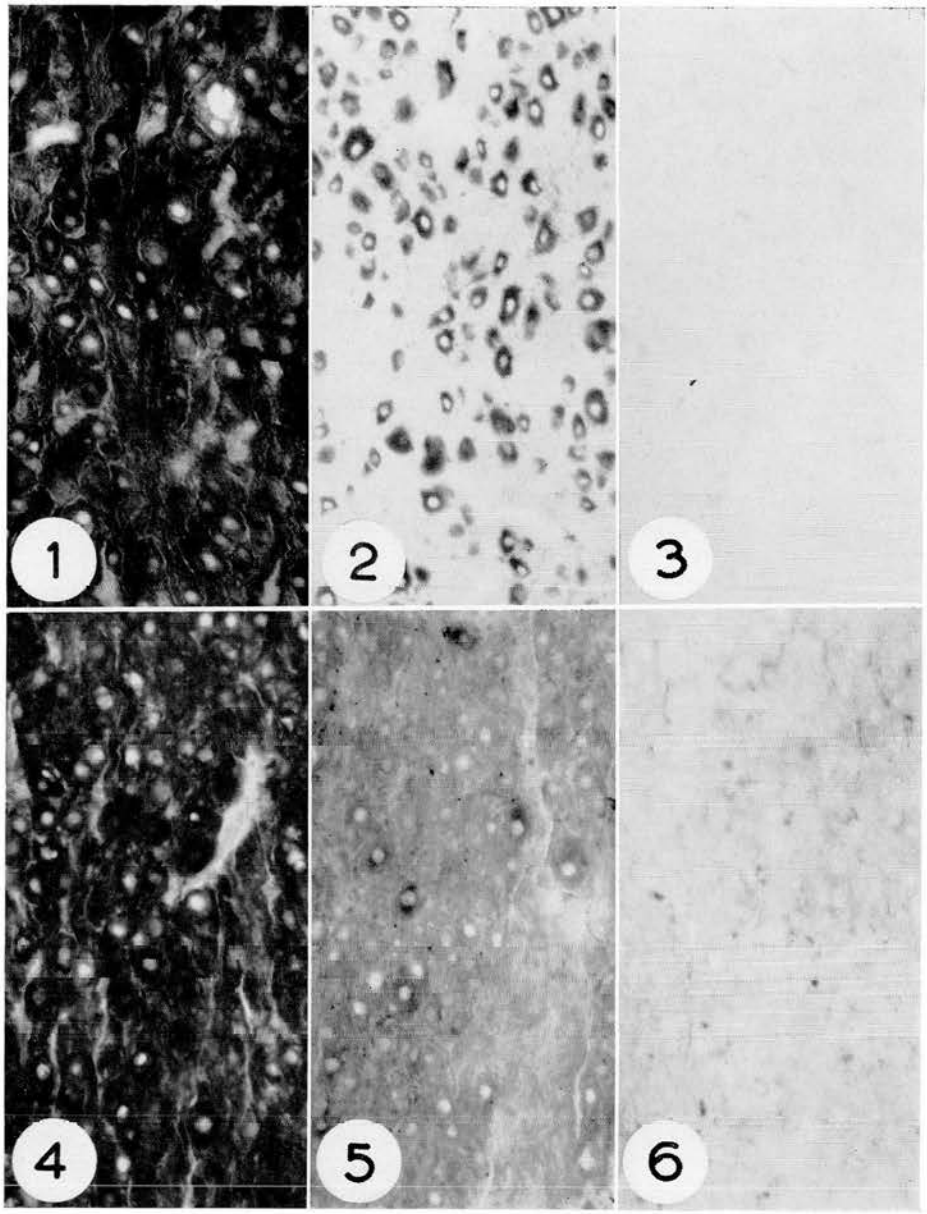
1. Distribution of esterase activity in sections

(a) *Sympathetic ganglion*

α-Naphthyl acetate. When this substrate was used without an inhibitor, a strong reaction was obtained practically throughout the sympathetic ganglion both in fresh (Fig. 1) and in formalin-fixed material (Fig. 4). Most of this activity was due to ns.ChE, since eserine or iso-OMPA equally abolished most of the activity (Figs. 2 and 5), while 284C51 had no effect. The distributions and respective contributions of AChE and ns.ChE to the total ChE activity were also studied using acetylthiocholine and iso-OMPA to demonstrate selectively AChE and butyrylthiocholine and 284C51 for ns.ChE. AChE, which was not demonstrable in sections with α-naphthyl acetate, gave a strong reaction in both ganglion cells and intercellular structures, while ns.ChE activity was mainly limited to structures between the ganglion cell bodies.

Comparison of Fig. 2 with Fig. 5 shows that the eserine resistant, i.e. ns.E, activity was clearly stronger in the ganglion cells of the fresh sections, while intercellular structures, which were entirely negative in fresh material, exhibited a moderate activity in formalin-fixed tissue.

All esterase activity was inhibited by E600 in fresh sections (Fig. 3). Although this inhibitor caused a considerable weakening in the intensity of the reaction also in most components of formalin-fixed material (Fig. 6), several elements, notably small cells between the ganglion cells still



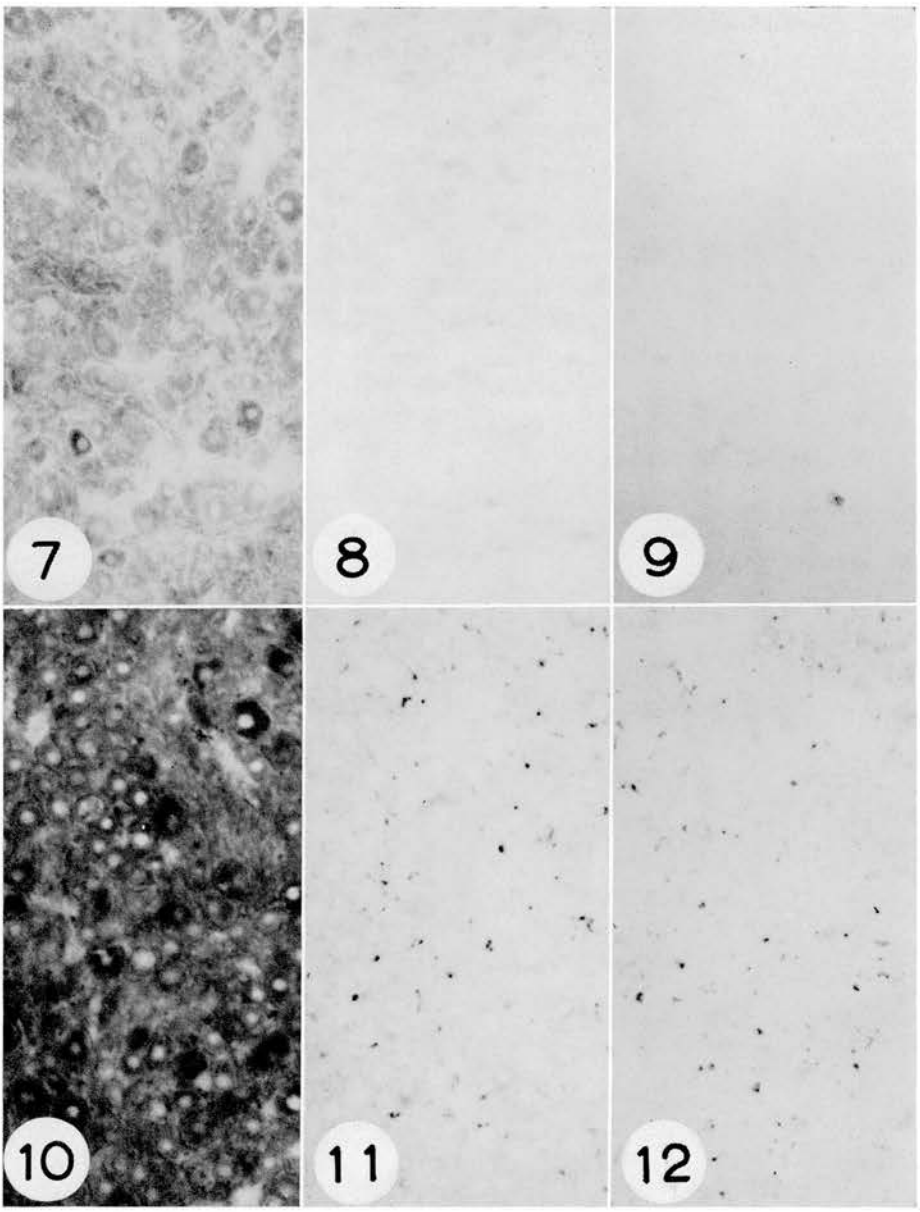
FIGS. 1–6. Esterase reactions obtained with α-naphthyl acetate in the sympathetic ganglion of the rat. (×100).

FIG. 1. Fresh section, no inhibitor
FIG. 2. Fresh section, 10^{-5} *M* eserine
FIG. 3. Fresh section, 10^{-5} *M* E600
FIG. 4. Formalin-fixed, no inhibitor
FIG. 5. Formalin-fixed, 10^{-5} *M* eserine
FIG. 6. Formalin-fixed, 10^{-5} *M* E600

exhibited a positive reaction, indicating E-r.ns.E activity.

4-Chloro-5-bromoindoxyl acetate. Without inhibitor, a positive reaction was obtained, as with alpha-naphthyl acetate, throughout the ganglion. However, when the incubation time was the same, the reaction was much weaker in fresh sections (Fig. 7) than in formalin-fixed material (Fig. 10),

although a rather similar picture was obtained also in fresh sections by prolonged incubation. Eserine abolished most activity from fresh sections (Fig. 8), indicating that the reaction obtained without inhibitor was mainly due to ChE activity. Since iso-OMPA had the same effect as eserine but 284C51 very little, it is seen that mainly ns.ChE was responsible. Also in formalin-

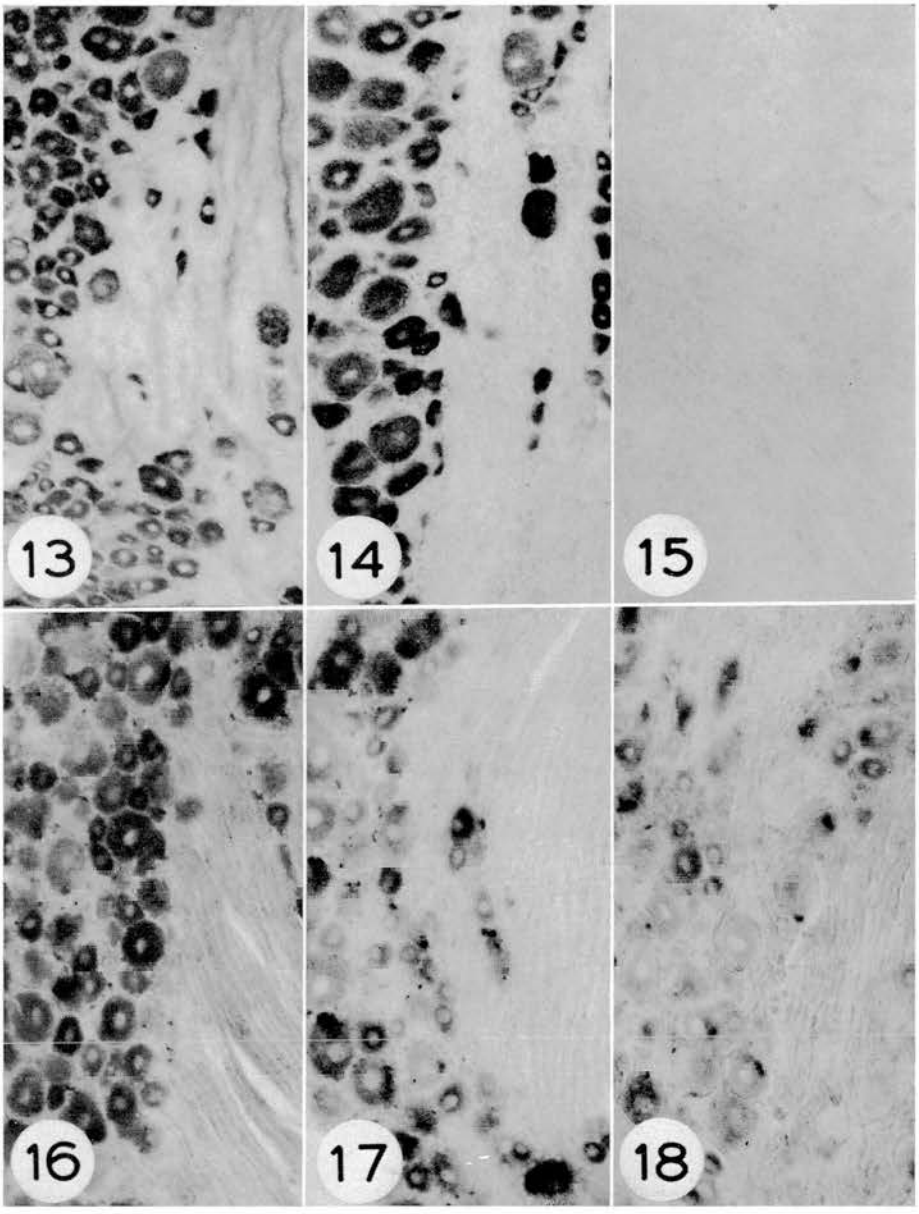


FIGS. 7–12. Esterase reactions obtained with 4-chloro-5-bromoindoxyl acetate in the sympathetic ganglion of the rat. (×100).

FIG. 7. Fresh section, no inhibitor
FIG. 8. Fresh section, 10^{-5} *M* eserine
FIG. 9. Fresh section, 10^{-5} *M* E600
FIG. 10. Formalin-fixed, no inhibitor
FIG. 11. Formalin-fixed, 10^{-5} *M* eserine
FIG. 12. Formalin-fixed, 10^{-5} *M* E600

fixed sections, eserine inhibited most of the activity but a strong reaction was still seen in the small cells (Fig. 11), which were also demonstrable with α-naphthyl acetate (Fig. 6).

The reaction in these cells was resistant not only to eserine but also to E600 (Fig. 12), and indeed there was very little difference between the reaction obtained with eserine and that obtained



FIGS. 13–18. Esterase reactions obtained with α-naphthyl acetate in the spinal ganglion of the rat. ($\times$100).

FIG. 13. Fresh section, no inhibitor
FIG. 14. Fresh section, 10^{-5} M eserine
FIG. 15. Fresh section, 10^{-5} M E600
FIG. 16. Formalin-fixed, no inhibitor
FIG. 17. Formalin-fixed, 10^{-5} M eserine
FIG. 18. Formalin-fixed, 10^{-5} M E600

with E600. Therefore, it seems that these cells contain mainly, if not exclusively, E-r.ns.E. In fresh sections these cells were not visible, both eserine (Fig. 8) and E600 (Fig. 9) inhibiting the reaction.

(*b*) *Spinal ganglion*

α-Naphthyl acetate. In fresh sections, the cytoplasm of the ganglion cells reacted strongly (Fig. 13), and this reaction was practically unchanged with eserine (Fig. 14), which on the other hand totally abolished the reaction from the nerve fibers; E600 entirely inhibited the reaction. In fresh sections of the spinal ganglion cells the reaction was therefore due only or almost only to ns.E, without ChE or E-r.ns.E activity.

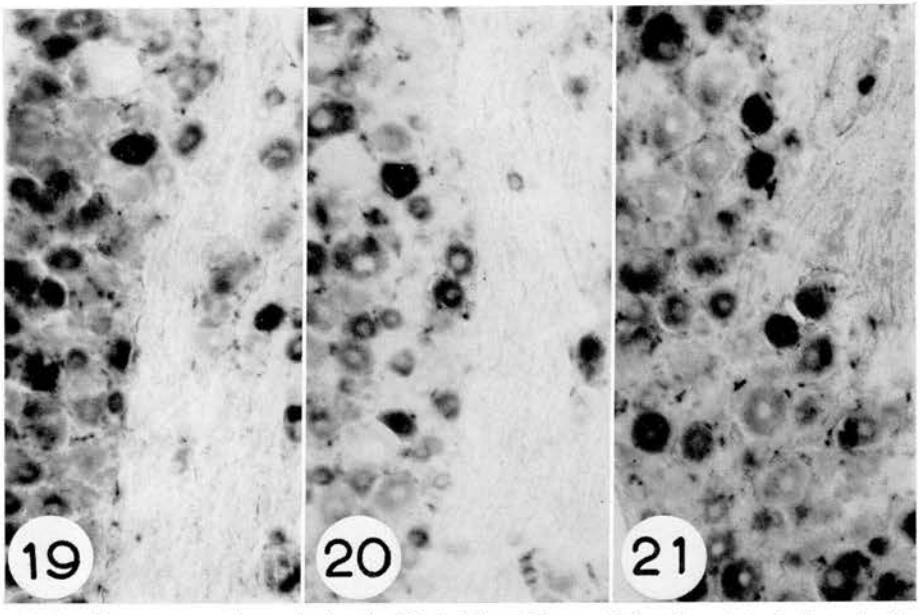
The same did not apply to fixed tissue. As compared with the activity observed without inhibitor (Fig. 16), most ganglion cells exhibited clearly diminished activity under the influence of eserine (Fig. 17), although a strong reaction was still seen in occasional cells. Since both iso-OMPA and 284C51 also decreased the intensity, the ChE activity demonstrable with this substrate can be seen to be composed of both AChE and ns.ChE activity. With acetylthiocholine it was possible to observe that the ChE activity of the ganglion cells was mainly AChE. E600 caused some further inhibition (Fig. 18), indicating the presence of some ns.E.

4-Chloro-5-bromoindoxyl acetate. Fresh sections of the spinal ganglion were so weakly reactive even without inhibitor that no photomicrographs were taken. The weak reaction persisted under the influence of eserine but was totally inhibited by E600.

In fixed material, a reaction whose intensity varied in individual ganglion cells was equally seen in sections developed without inhibitor (Fig. 19), with eserine (Fig. 20) or with E 600 (Fig. 21), indicating that most of the enzyme activity was due to E-r.ns.E. Small cells between the ganglion cells also exhibited a strong reaction of this kind.

2. Starch gel electrophoresis of tissue homogenates

Fig. 22 shows zymograms obtained from homogenates of the sympathetic ganglion (the left-hand group) and the spinal ganglion (the right-hand group) with the following representative substrates: α-naphthyl acetate (N), naphthol AS-D acetate (A), 4-chloro-5-bromoindoxyl acetate (I) and acetylthiocholine (T). Since the main purpose of the present report is to compare enzyme activities in sections and in starch slabs, rather than to map and characterize each individual band in the zymograms, only a general account



FIGS. 19–21. Esterase reactions obtained with 4-chloro-5-bromoindoxyl acetate in the spinal ganglion of the rat. (×100).

FIG. 19. Formalin-fixed, no inhibitor
FIG. 20. Formalin-fixed, 10^{-5} *M* eserine
FIG. 21. Formalin-fixed, 10^{-5} *M* E600

of the band characteristics will be given. In so doing, designations ChE, ns.E and E-r.ns.E are used, according to the effect of eserine and E600.

The effect of these inhibitors is not actually demonstrated in Fig. 22 but the zymograms are divided into zones according to the results of the inhibitor experiments carried out, using abbreviations "Ch" for ChE, "ns" for ns.E and "E-r" for E-r.ns.E. A further region exhibited all of the three types of activity, partly in narrow bands with only one type of activity, partly in bands at the same time containing ChE, ns.E and E-r. ns.E. This region was marked "mix". It must also be noted that while the regions ns, E-r 1 and E-r 2 were pure, a minor but clear part of the total activity of the Ch zone was due to ns.E, although it was given this name because of predominant ChE activity. The activity in this zone was destroyed by formalin fixation for 1 hour, although the other bands were but little affected.

The mix area represented only a small proportion of either ChE or ns.E activity, most of which was present in the areas Ch and ns, respectively. Therefore, the activities of these areas can alone be used to obtain an approximate idea of the total ChE and ns.E activities in the slab. However, because a considerable proportion of the total E-r.ns.E was present in the mix area, besides the pure areas E-r 1 and 2, the mix area must be taken into consideration in assessing this type of activity.

Of the substrates employed, α-naphthyl acetate was the only substrate showing all the bands demonstrable with any of the other substrates, which were more selective, as can be seen from Fig. 22. As was shown by experiments with inhibitors (not illustrated here), naphthol-AS-D acetate (A) demonstrated ns.E and E-r.ns.E activity but hardly any ChE activity, 4-chloro-5-bromoindoxyl acetate (I) gave positive reactions for ns.ChE and E-r.ns.E but showed practically no ns.E and very little AChE activity, and acetylthiocholine (T) gave positive reactions with ChEs alone. It is noteworthy that although alpha-naphthyl acetate demonstrated also AChE activity in zymograms, this activity was not as widely spread in the zymograms as that demonstrable with acetylthiocholine.

3. Comparison of esterases in zymograms of homogenates and in sections

The relative proportions of ChE, ns.E and E-r.ns.E of the total esterase activity were different in zymograms of homogenates and in fresh sections. In sections of the sympathetic ganglion, e.g., a great majority of the total activity towards α-naphthyl acetate was eserine sensitive (Fig. 2), while in the zymograms of the same organ most of the activity towards the same substrate was due to ns.E and E-r.ns.E activity resistant to eserine. Discrepancies between sections and zymograms were observed also using other substrates and inhibitors.

This suggests that the enzymes demonstrable in fresh sections are different from the enzymes responsible for the positive bands in the zymograms. Indeed, strong reactions were regularly observed in the filter paper to which the supernatant of the centrifuged homogenate was applied and also in the gel at the origin in all zymograms but those developed in the presence of E600. It is therefore evident that only a part of the enzymes were capable of moving along the gel.

4. Solubility of esterases in fresh unfixed sections

The effect of distilled water was first examined by treating fresh-frozen sections which were first allowed to dry on slides with water for varying periods. Immersion in water for up to 30 min. caused no detectable effect on the intensity or distribution of esterase activity histochemically demonstrable with α-naphthyl acetate, although the cytological preservation was poor. This indicates that the reaction obtained with this substrate in fresh sections is due principally or exclusively to poorly soluble esterases, i.e. desmo-esterases.

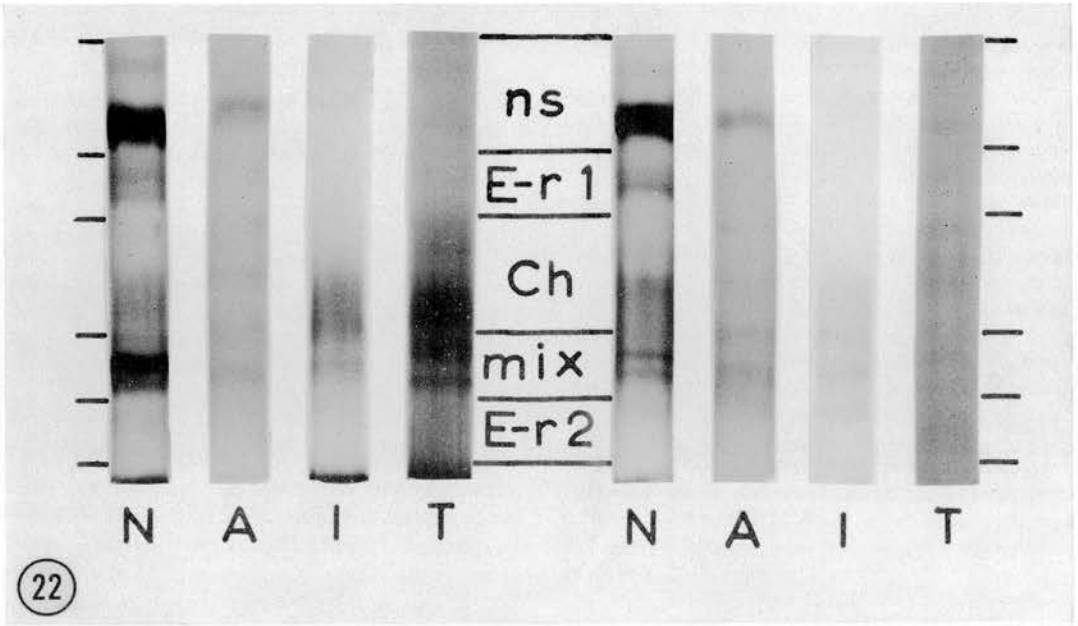
The soluble extracts derived from fresh sections that had been covered with water for 1 min, on the other hand, were observed to produce zymograms which were almost similar to those obtained with homogenates with about the same tissue concentration (cf. the first zymogram of Fig. 23 with the first zymogram of Fig. 22). This shows that the esterases responsible for the activity in the zymograms are readily soluble in water, i.e. lyo-esterases.

It thus seems clear that lyo-enzymes mainly contribute to the esterase activity in the zymograms and desmo-esterases to that demonstrable in the sections. Moreover, lyo-esterases seem to be very soluble and desmo-esterases very insoluble.

5. Electrophoretic properties of lyo- and desmo-esterases

Lyo- and desmo-zymograms were prepared from the same set of sections as described previously in the present paper.

In Fig. 23 are illustrated lyo- (L) and desmo-zymograms (D) obtained with α-naphthyl acetate of the sympathetic ganglion (the left-hand group) and the spinal ganglion (the right-hand group).



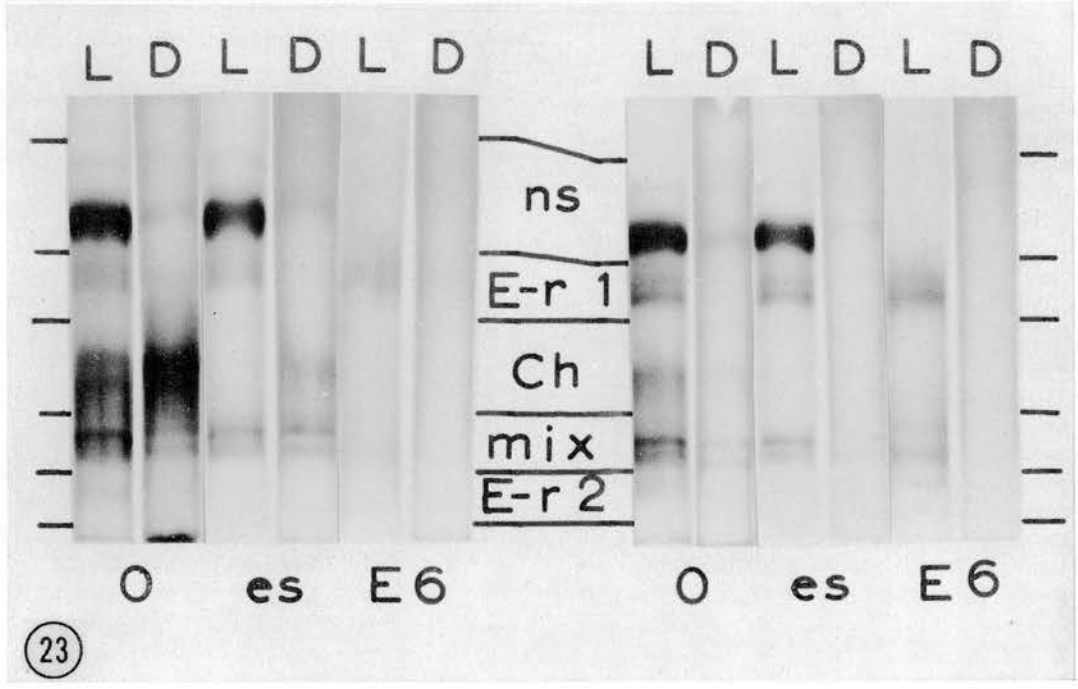


FIG. 22. Homogenate zymograms of the sympathetic ganglion (left group) and the spinal ganglion (right group) of the rat, obtained with α-naphthyl acetate (N), naphthyl-AS-D acetate (A), 4-chloro-5-bromoindoxyl acetate (I) and acetylthiocholine (T). Regions of different types of esterases indicated in the centre: ns, ns.E; E-r, E-r.ns.E; Ch, ChE; mix, area with several types of activity.

FIG. 23. Lyo-(L) and desmo-(D) zymograms of the sympathetic ganglion (left group) and the spinal ganglion (right group) of the rat, obtained with α-naphthyl acetate without inhibitor (0), with 10^{-5} *M* eserine (es) and with 10^{-5} *M* E600 (E6). Areas of different types of esterase activity indicated in the centre as in Fig. 22.

The first pair in each group was developed without inhibitor (0), the second with eserine (es) and the third with E600 (E6) as an inhibitor.

The total enzyme activity of the lyo-zymogram was always more intense than that of the desmo-zymogram. However, this is partly due to the fact that not all of the total desmo-activity in the sections was liberated by freezing, thawing and homogenization but remained attached to the tissue fragments removed by centrifugation. Furthermore, a part of the activity recovered in the supernatant was not mobile and remained in or near the origin during electrophoresis (see second zymogram, 0, D in Fig. 23). The L-D pairs can therefore be mainly used for the qualitative comparison of the enzyme spectra only.

No E-r.ns.E activity was observed in the desmo-zymograms. Since such activity was neither demonstrated at or near the origin of desmo-zymograms or zymograms made of whole homogenates, it appears that all E-r.ns.E belongs to the lyo-fraction, which explains that no E-r.ns.E activity can be demonstrated in fresh sections (Figs. 3, 9 and 15).

A major part of the ns.E activity likewise appears to be of the lyo type, because very little activity was found in the ns region of the desmo-zymograms, where an intense reaction was observed in the lyo-zymograms (cf. L and D in pairs marked es in Fig. 23). It is therefore of special interest that the ns.E activity of the sympathetic ganglion in the Ch zone, earlier mentioned in the description of the homogenate zymograms, was clearly demonstrable in the desmo-zymogram but not in the lyo-zymogram. In the spinal ganglion, ns.E activity was practically limited to the ns zone only.

In contrast to ns.E and E-r.ns.E activities, a major part of the ChE activity of the sympathetic ganglion was obviously of the desmo type, because the eserine sensitive reaction of the Ch zone was stronger in the desmo-zymogram than that in the lyo-zymogram (extreme left 0 pair in Fig. 23). This is the more significant, because a further part of the desmo-ChE activity remained attached to tissue or did not move along the gel. In the spinal ganglion, on the contrary, notable ChE activity was observed only in the lyo-zymogram (the 0 pair of the right-hand group in Fig. 23). This explains why eserine abolished most of the histochemical reaction in fresh sections of the sympathetic ganglion (cf. Figs. 1 and 2) but had little effect on the reaction in sections of the spinal ganglion (cf. Figs. 13 and 14).

6. Comparison of esterases in desmo-zymograms and sections

In contrast to zymograms of homogenates and lyo-zymograms, the desmo-zymograms contained about the same proportions of ChE, ns.E and E-r.ns.E of the total esterase activity as was observed in fresh sections. In the sympathetic ganglion both desmo-zymograms and fresh sections exhibited much ChE, little ns.E and no E-r.ns.E activity. In the spinal ganglion, most of the total activity was due to ns.E, and there was very little ChE and no E-r.ns.E either in the sections or the desmo-zymograms.

DISCUSSION

The observations of the present work strongly suggest that only desmo-enzymes can be histochemically demonstrated in fresh sections, since lyo-enzymes dissolve away before the histochemical reaction has had time to occur. On the other hand, the data presented indicates that starch gel electrophoresis carried out using a tissue homogenate as a starting material is a poor indicator of the variety of enzymes histochemically demonstrable in fresh sections, because the zymogram pattern obtained is mainly determined by lyo-enzymes, which do not contribute to the histochemical reaction in fresh sections.

Several earlier studies have indicated that esterases are to a variable extent bound to tissue sections and that only a part of the original enzyme activity is therefore responsible for histochemical reactions obtained in sections (1, 3, 8, 13, 19). Moreover, circumstantial evidence has been presented to show that the substrate-inhibitor characteristics of soluble and poorly soluble esterases are at least partly different (1, 8, 9, 13, 19). Studies carried out with starch gel electrophoresis show that the esterase activity of tissues is due to the simultaneous action of several esterases differing from each other in many respects (1, 3, 6, 8, 9, 16, 18). As far as we know, the present work has for the first time provided a direct proof of the differences in the enzymatic properties of lyo- and desmo-esterases by showing that their zymogram patterns are dissimilar in many respects.

Not only are there many different types of esterases but not all of them can be demonstrated histochemically, either separately or simultaneously. This makes the study of esterases more

complicated. Fortunately, the same observations which demonstrate an almost bewildering complexity of contributing factors also provide some clues about the possibilities of differentiating the many esterases.

The observations of the present study suggest that esterases responsible for the histochemical reaction in fresh sections are fairly well represented in desmo-zymograms, which makes it possible to characterize them by using different substrate-inhibitor combinations. On the other hand, it appears clear that nothing can be said of the lyo-enzymes by examination of fresh sections, in which only desmo-enzymes are present. All E-r.ns.E and a major part of the total ns.E was found to belong to the lyo-group, and although a major part of ChE activity was observed in the desmo-group, a considerable part of ChE activity was soluble.

Since fresh sections, therefore, give a very incomplete idea of the total esterase activity, the question arises how far it is possible to immobilize the lyo-esterases and thus make them accessible for histochemical demonstration. The results of the present study indicate that simple formalin fixation brought about a positive esterase reaction in many such areas which were entirely negative in fresh sections. Thus, E-r.ns.E, which was not at all demonstrable in fresh sections, exhibited in formalin-fixed material a strong reaction in certain structures of the sympathetic (Figs. 6 and 12) and spinal (Figs. 18 and 21) ganglia. Another example is the intense reaction due to ns.ChE obtained with 4-chloro-5-bromoindoxyl acetate in formalin-fixed (Fig. 10) but not in fresh (Fig. 7) sympathetic ganglion.

Under favorable circumstances, differences in solubility may make it possible to identify a histochemical reaction in sections with a specific area in the zymogram. In fresh sections of the sympathetic ganglion, ns.E activity was limited to the cytoplasm of the ganglion cells (Fig. 2). Since most of the total ns.E activity in the desmo-zymogram was limited to the Ch region (Fig. 23, es, D, left group), it is reasonable to ascribe the ns.E activity of the ganglion cells to the ns.E activity in this region.

On the other hand, formalin fixation brought about a positive ns.E reaction in the intercellular tissue (Fig. 5), which is non-reactive in fresh sections (Fig. 2). Assuming that the effect of formalin depended on immobilization of lyo-esterases and since most of the ns.E activity of the lyo-zymograms was in the ns zone (Fig. 23, es, L, left group), it appears that the intercellular reaction of the fixed tissue was due to enzymes responsible for the two bands in this zone.

Actually, the effect of formalin is not limited to immobilization only. It also inactivates, and apparently some esterases are more affected than the others. Thus, the reaction towards α-naphthyl acetate in the Ch region of homogenate zymograms was completely destroyed by formalin, although the activity of the ns region was hardly affected by it. It is therefore likely that the ns.E responsible for the reaction in the cytoplasm of the sympathetic ganglion cells of fresh sections (Fig. 2) was destroyed by formalin and did not contribute at all to the cytoplasmic reaction visible in fixed material (Fig. 5), which probably was due to the same lyo-ns.E which also brought up the intercellular reaction.

Since formalin at the same time immobilizes some enzymes and destroys others, it is possible that sections of fresh and fixed material look almost similar, although different enzymes are responsible for the reactions seen in them. Thus, a positive reaction towards α-naphthyl acetate was observed in the cytoplasm of the spinal ganglion cells both in fresh (Fig. 13) and in formalin-fixed (Fig. 16) material. However, only a weak reaction was observed with 4-chloro-5-bromoindoxyl acetate in fresh sections of the spinal ganglion, although the reaction towards this substrate obtained in fixed material (Fig. 19) was essentially similar to that obtained with α-naphthyl acetate.

An adequate explanation can be provided by examining the nature of the esterases responsible. In fresh sections of the spinal ganglion, practically all activity was due to desmo-ns.E (cf. Figs. 13, 14 and 15, as well as the D zymograms 0, es and E6 in the right-hand group of Fig. 23). In contrast to α-naphthyl acetate, 4-chloro-5-bromoindoxyl acetate is a poor substrate for both AChE and ns.E (cf. zymograms N and I in Fig. 22). Accordingly, it provided but a weak reaction with fresh sections, whose activity was mainly due to desmo-ns.E. Formalin fixation destroyed this activity (cf. Fig. 14 and Fig. 17) but immobilized lyo-esterases exhibiting ChE, ns.E and E-r.ns.E activity, all of which are demonstrable

with α-naphthyl acetate (Figs. 16, 17 and 18). Of them only ns.ChE and E-r.ns.E are well demonstrable with 4-chloro-5-bromoindoxyl acetate, and since there is very little ns.ChE in the spinal ganglion, the reaction obtained with this substrate in fixed ganglia was principally due to E-r.ns.E (Figs. 19, 20 and 23).

The above examples may be sufficient to illustrate the possibilities of using information obtained by examining the esterase activity of a tissue both in fresh and fixed sections and in desmo- and lyo-zymograms to characterize the different types of esterase activity. It seems to us that this approach not only makes it possible to analyse different types of esterase activity in a way not otherwise possible, but is, indeed, necessary unless one is content with a tinctorial effect of an esterase reaction without regard to the nature of the esterases contributing to it.

It must be pointed out that the presentation in the present paper is simplified and only endeavours to illustrate the value of the conjoint use of several techniques each providing some special information, without attempting to give a complete analysis of the esterases of the spinal and sympathetic ganglion. It must also be emphasized that the methodological set-up does not cover the whole esterase spectrum of these tissues. Although information was obtained about the desmo-enzymes by studying electrophoretically esterases liberated from sections by freezing and thawing, a considerable part of the desmo-esterase activity still remained attached to tissue and thus escaped further analysis.

However, recent studies (13, 19) indicate that there are possibilities both for improving the solubility of desmo-enzymes by using proper extracting solutions and for improving the fixation of lyo-enzymes by modifying the composition of conventional fixation mixtures. It would therefore seem likely that further efforts would lead to improved characterization of the chemical nature of desmo-enzymes, on the one hand, and to improved localization of lyo-enzymes in tissue sections, on the other.

To conclude, then, the present work has provided evidence suggesting that esterases form a family of enzymes which differ from each other not only in substrate and inhibitor characteristics, but also in electrophoretic mobility and solubility. In studying the functional significance of esterases due attention should therefore be paid to this multiplicity by making use of as many independent techniques as possible to characterize the nature of the enzyme activity whose function is under investigation.

REFERENCES

1. Allen, J. M., Eränkö, O., and Hunter, R. L. A histochemical study of the esterases of the adrenal medulla of the rat. *Am. J. Anat.* **102:** 93–116, 1958.
2. Barron, K. D., Bernsohn, J. I., and Hess, A. Starch gel electrophoresis of brain esterases. *J. Histochem. Cytochem.* **9:** 656–660, 1961.
3. Barron, K. D., Bernsohn, J. I., and Hess, A. Separation and properties of human brain esterases. *J. Histochem. Cytochem.* **11:** 139–156, 1963.
4. Bayliss, B. J., and Todrick, A. The use of a selective acetylcholinesterase inhibitor in the estimation of pseudocholinesterase activity in rat brain. *Biochem. J.* **62:** 62–67, 1956.
5. Eränkö, O. Specific demonstration of acetylcholinesterase and non-specific cholinesterase in the adrenal gland of the rat. *Histochemie* **1:** 257–266, 1959.
6. Eränkö, O., Kokko, A., and Söderholm, U. Separation of substrate-specific brain esterases by starch-gel electrophoresis. *Nature* **193:** 778–779, 1962.
7. Eränkö, O., Kokko, A., and Söderholm, U. Histochemical evidence of three types of esterases in the adrenal medulla of the rat. *Histochemie* **2:** 383–388, 1962.
8. Eränkö, O., Kokko, A., and Söderholm, U. Starch electrophoretic separation of esterases in the adrenal medulla, adrenal cortex and the brain of the rat and the hamster. *Ann. Med. Exper. Biol. Fenniae* **40:** 146–161, 1962.
9. Esilä, R. Histochemical and electrophoretic properties of cholinesterases and non-specific esterases in the retina of some mammals, including man. *Acta Ophthalmol.* **41:** Suppl. 77: 1–113, 1963.
10. Gomori, G. *Microscopic Histochemistry. Principles and Practice.* Univ. of Chicago Press, Chicago, 1952.
11. Gomori, G., and Chessick, R. D. Histochemical studies of the inhibition of esterases. *J. Cell. Comp. Physiol.* **41:** 51–64, 1953.
12. Gössner, W. Histochemischer Nachweiss hydrolytischer Enzyme mit Hilfe der Azofarbstoffmethode. *Histochemie* **1:** 48–96, 1958.
13. Hannibal, M. J., and Nachlas, M. M. Further studies on the lyo and desmo components of several hydrolytic enzymes and their histochemical significance. *J. Biophys. Biochem. Cytol.* **5:** 279–288, 1959.
14. Holmstedt, B. A modification of the thiocholine method for the determination of cholinesterase. II. Histochemical application. *Acta Physiol. Scand.* **40:** 331–337, 1957.
15. Holt, S. J. Indigogenic staining methods for esterases. In *General Cytochemical Methods, Vol.* **1.** (Ed. J. F. Danielli). Academic Press, Inc., New York, 1958, 375–398.

16. HUNTER, R. L., AND BURSTONE, M. S. The zymogram as a tool for the characterization of enzyme substrate specificity. *J. Histochem. Cytochem.* **8:** 58–62, 1960.
17. KOELLE, G. B. The elimination of enzymic artefacts in the histochemical localization of cholinesterases and survey of their cellular distributions. *J. Pharmacol. Exper. Therap.* **103:** 153–171, 1951.
18. MARKERT, C. L., AND HUNTER, R. L. The distribution of esterases in mouse tissues. *J. Histochem. Cytochem.* **7:** 42–49, 1959.
19. NACHLAS, M. M., PRINN, W., AND SELIGMAN, A. M. Quantitative estimation of lyo- and desmoenzymes in tissue sections with and without fixation. *J. Biophys. Biochem. Cytol.* **2:** 487–502, 1956.
20. PEARSE, A. G. E. *Histochemistry, Theoretical and Applied.* Churchill, London, 1953.
21. PEPLER, W. J., AND PEARSE, A. G. E. The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei. *J. Neurochem.* **1:** 193–202, 1957.
22. SHNITKA, T. K., AND SELIGMAN, A. M. Role of esteratic inhibition on localization of esterase and the simultaneous cytochemical demonstration of inhibitor sensitive and resistant enzyme species. *J. Histochem. Cytochem.* **9:** 504–525, 1961.

Acta physiol. scand. 1965. 63. 411—412

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Effect of Axon Division on the Distribution of Noradrenaline and Acetylcholinesterase in Sympathetic Neurons of the Rat

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

The distribution of histochemically demonstrable noradrenaline (NA) suggests that this amine is synthetized in the cytoplasm of sympathetic ganglion cells and migrates along the axon towards the periphery (Eränkö and Härkönen 1963). To test this possibility, the effect of nerve division was examined in the present study. Besides the distribution of NA, that of acetylcholinesterase (AChE) was studied, because previous work showed that many sympathetic cells with a high NA content also exhibit an intense AChE activity (Eränkö and Härkönen 1964).

Pre- or postganglionic nerves of the superior cervical ganglion of the rat were divided a few mm from the ganglion. After a period ranging from 1 day to 3 months, the ganglion and the nerve stumps attached to it were removed and freeze-dried. NA was demonstrated with formaldehyde-induced fluorescence (Eränkö 1964) and AChE activity with Gomori's (1962) modification of Koelle's thiocholine method. Non-specific cholinesterase was inhibited with tetraisopropylpyrophosphoramide (iso-OMPA).

Division of the preganglionic nerve trunk had no marked effect on NA content or AChE activity of the ganglion cells, although it caused a virtually total loss of AChE activity from the preganglionic fibres and synapses.

Postganglionic denervation resulted in a loss of both NA and AChE activity from the ganglion cells. Very little AChE activity was seen in these cells 2 days after the operation, while the loss of NA was slower, reaching a maximum in about a week. However, accumulation of NA in the proximal nerve stump was clearly demonstrable already after 2 days (Fig. 1). AChE activity of the stump was also increased. The small, intensely fluorescent cells normally present in the ganglion (Eränkö and Härkönen 1963) were not affected by nerve division. NA content and AChE activity of the ganglion cells gradually increased 1—2 months after denervation, reaching the normal level in about 3 months.

The observations presented indicate that NA and AChE indeed migrate from the cell body to the axon, after this has been divided. Dahlström and Fuxe (1964) have in a recent study, published after the present work was completed, independently arrived

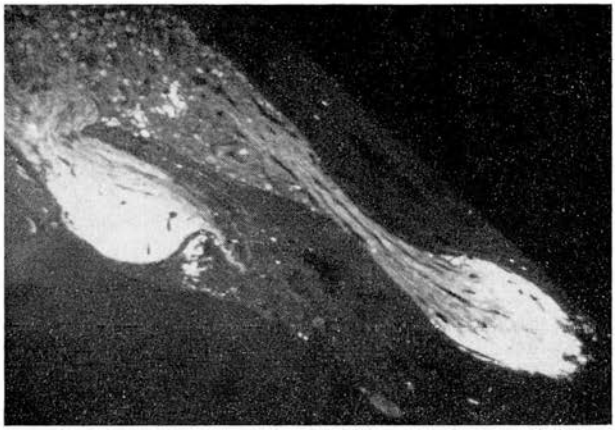


Fig. 1. Distribution of formaldehyde-induced fluorescence in the sympathetic ganglion and two postganglionic nerve trunks 2 days after division of the latter. The fluorescence of the ganglion cell bodies (left upper corner) is weak, that in the ends of the nerve stumps is extremely intense. Small, intensely fluorescent cells are visible in the ganglion between the nerve trunks. Such cells are normally present in the ganglion. 30 ×.

at the same result concerning NA and made use of it to demonstrate monoamine-containing fibres in the central nervous system.

It appears likely that axonic flow of NA and AChE occurs not only after nerve division but also in an intact neuron. However, this does not exclude the possibility of their uptake or synthesis in the peripheral parts of the nerve fibre (see de Robertis 1964).

References

Dahlström, A. and K. Fuxe, A method for the demonstration of monoamine-containing nerve fibres in the central nervous system. *Acta physiol. scand.* 1964. *60.* 293—294.

De Robertis, E., *Histophysiology of Synapses and Neurosecretion.* 1964. Pergamon Press, Oxford

Eränkö, O., Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.* 1964. *12.* 487—488.

Eränkö, O. and M. Härkönen, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58.* 285—286.

Eränkö, O. and M. Härkönen, Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1964. *61.* 299—300.

Gomori, G., *Microscopic Histochemistry, Principles and Practice.* 1952. The University of Chicago Press, Chicago.

KUNGL. BOKTR. STHLM 1965

EXCERPTUM

Acta physiol. scand. 1965. 63. 505—506

From the Department of Anatomy, University of Helsinki, Finland

Fibres Containing both Noradrenaline and Acetylcholinesterase in the Nerve Net of the Rat Iris

By

Olavi Eränkö and Liisa Räisänen

Ganglion cells which contain both noradrenaline (NA) and acetylcholinesterase (AChE) have been demonstrated in the superior cervical ganglion of the rat (Eränkö and Härkönen 1964). In the present report, observations are presented showing that NA and AChE are present together also in some fine fibres of the sympathetic nerve net of the iris.

The iris was spread fresh on a clean slide and was allowed to dry. NA and AChE were then demonstrated in each preparation, as described recently (Eränkö 1964). The dry iris was first exposed to formaldehyde vapour to render NA fluorescent and photographed. It was then subjected to Gomori's (1952) modification of Koelle's cholinesterase method and photographed again.

As applied to iris, the method proved difficult, because formaldehyde tended to destroy all AChE activity in it. However, by reducing the exposure to formaldehyde just long enough to induce NA fluorescence, about 20 min at 40° C, AChE activity was still demonstrable thereafter.

The distributions of NA and AChE were compared by superimposing the pairs of photomicrographs made of each iris preparation. It was thus possible to compare the two reactions in the fine tortuous fibres more easily than in pictures examined side by side. Fibres with NA appeared white against dark background in the fluorescence photograph and those with AChE activity appeared dark against light background in the other photograph. Accordingly, fibres which were white in one picture and dark in the other disappeared when accurately superimposed.

Many of the finest fibres of the iris, obviously belonging to the terminal sympathetic nerve net, not only exhibited fluorescence due to NA but also showed AChE activity. Other fibres were fluorescent but did not exhibit AChE activity and *vice versa*. This suggests that the innervation apparatus of the iris includes 3 types of fibres, *i. e.* fibres containing both NA and AChE and fibres containing either NA or AChE.

Confirming our preliminary observations on the iris (Eränkö and Härkönen 1964), the results now presented further strengthen the hypothesis of the existence of sympathetic fibres which are at the same time adrenergic and cholinergic (Burn and Rand 1962, Koelle 1962). They also complement Richardson's (1964) recent electron microscopic observations, according to which some synapses of the iris, presumably the cholinergic ones, contain synaptic vesicles only, while others, presumably the adrenergic

ones, contain both synaptic vesicles and electron-dense, probably catecholamine-containing droplets.

However, Richardson (1964) also showed that fine nerve fibres often run so close together that they probably appear as one fibre under the light microscope. The demonstration in the present work of both NA and AChE apparently in single fibres may therefore be due to closely associated but separate fine fibres, some of which contain NA and others which exhibit AChE activity. In view of the existence of ganglion cells containing both NA and AChE in the superior cervical ganglion (Eränkö and Härkönen 1964), from which originate the sympathetic fibres of the iris, the real existence of similar peripheral fibres appears more likely. Even if adrenergic and cholinergic fibres of the iris should be separate, they appear to be close enough to make possible the interaction of cholinergic and adrenergic mechanisms as proposed by Burn and Rand (1962).

During Professor G. B. Koelle's visit to our laboratory in August 1964 it became apparent that observations essentially similar to those reported in the present paper have independently been made in his laboratory in the University of Pennsylvania. We gratefully acknowledge stimulating discussions with him.

References

Burn, J. H. and M. J. Rand, A new interpretation of the adrenergic nerve fiber. In *Adv. Pharmacol.* (Ed. S. Garattini and P. A. Shore). Vol. *1*. 1—30. Academic Press, New York and London. 1962.

Eränkö, O., Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.* 1964. *12*. 487—488.

Eränkö, O. and M. Härkönen, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58*. 285—286.

Eränkö, O. and M. Härkönen, Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1964. *61*. 299—300.

Gomori, G., *Microscopic Histochemistry, Principles and Practice*. The University of Chicago Press. Chicago. 1952.

Koelle, G. B., A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. *J. Pharm. Pharmacol.* 1962. *14*. 65—90.

Richardson, K. C., The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles. *Amer. J. Anat.* 1964. *114*. 173—205.

KUNGL. BOKTR. STHLM 1965

EXCERPTUM

Acta physiol. scand. 1965. 63. 511—512

From the Department of Anatomy, University of Helsinki, Finland

Monoamine-Containing Small Cells in the Superior Cervical Ganglion of the Rat and an Organ Composed of Them

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

Small cells have been observed in the superior cervical ganglion of the rat which exhibit an extremely bright yellow fluorescence after exposure to formaldehyde (Eränkö and Härkönen 1963, see also Fig. 1 in Eränkö and Härkönen 1965). The present report describes some properties of these cells and an organ composed of them.

Freeze-dried ganglia were exposed to formaldehyde, as described previously (Eränkö 1964). The fluorescence in the small cells was not only brighter and of different colour than that in the ganglion cells but it was also resistant to treatment with distilled water, while breathing once on the section entirely abolished the green fluorescence of the ganglion cells. After staining in 1 % thionin, which did not either destroy the fluorescence in the small cells (Fig. 1), no colour was seen in the cytoplasm (Fig. 2). This shows that the cells were not mast cells, which is of interest because these also exhibit an intense yellow fluorescence resistant to water (unpublished observations; see also Lagunoff *et al.* 1961).

Complete series of sections were prepared from ganglia fixed in 3.5 % potassium dichromate. No or few chromaffin cells were found in each ganglion. This indicates that the far more numerous small cells must be non-chromaffin. It is therefore somewhat surprising that cells of the same size and shape as the strongly fluorescent ones were electron microscopically observed to closely resemble the chromaffin cells of the adrenal medulla, containing, like these, numerous intensely osmiophilic granules (Eränkö and Hänninen, to be published).

It thus seems that the small cells contain high concentrations of a monoamine, perhaps 5-hydroxytryptamine, apparently stored in secretory granules in the same manner as catecholamines in the adrenal medulla. They represent a new variety of non-chromaffin amine-storing cells.

While the small cells were scattered in the superior cervical ganglion, especially near and in nerve bundles, a well vascularized organ composed of these cells was accidentally observed near the ganglion (Fig. 3). The organ was first thought to be the carotid body but this was subsequently found to be composed of cells exhibiting a green, less intense fluorescence. Therefore, the small cells apparently form a hitherto unknown endocrine gland which perhaps secretes 5-hydroxytryptamine.

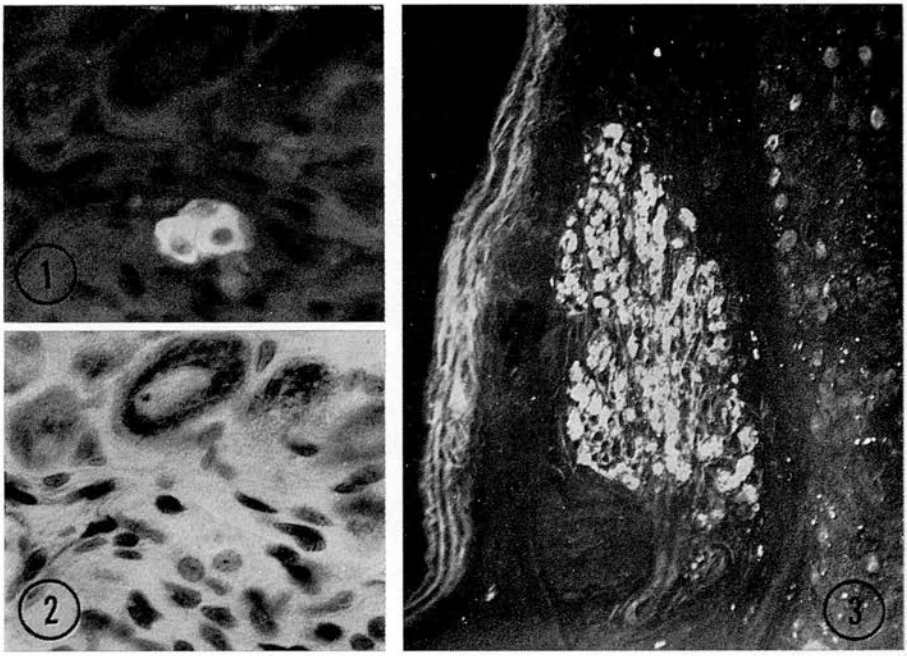


Fig. 1. Fluorescence photomicrograph of a freeze-dried ganglion exposed to formaldehyde and stained with 1 % thionin. Two small cells still fluoresce intensely. 600 ×.

Fig. 2. Same field as in Fig. 1 photographed in transmitted visible light. Ganglion cells and nuclei are stained but the cytoplasm of the two small cells is unstained. 600 ×.

Fig. 3. Organ composed of the small intensely fluorescent cells in the centre. On the left, wall of an artery. On the right, the superior cervical ganglion. Formaldehyde-induced fluorescence. 75 ×.

References

ERÄNKÖ, O., Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.* 1964. *12*. 487—488.

ERÄNKÖ, O. and M. HÄRKÖNEN, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58*. 285—286

ERÄNKÖ, O. and M. HÄRKÖNEN, Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. *Acta physiol. scand.* 1965. *63* 411—412.

LAGUNOFF, D., M. PHILLIPS and E. V. BENDITT, The histochemical demonstration of histamine in mast cells. *J. Histochem. Cytochem.* 1961. *9*. 534—541.

KUNGL. BOKTR. STHLM 1965

Reprinted from
MECHANISMS OF RELEASE OF BIOGENIC AMINES
Proceedings of an International Wenner-Gren Symposium held in Stockholm, February 1965
PERGAMON PRESS
OXFORD · LONDON · EDINBURGH · NEW YORK
TORONTO · PARIS · BRAUNSCHWEIG
1966

IN VITRO RELEASE AND UPTAKE OF NORADRENALINE IN THE RAT IRIS

OLAVI ERÄNKÖ and LIISA RÄISÄNEN
Department of Anatomy, University of Helsinki,
Siltavuorenpenger, Helsinki

NERVE fibres whose intrinsic stores of noradrenaline (NA) have been depleted with reserpine rapidly restore the normal NA content after exogenous administration of this amine (Hillarp and Malmfors, 1964). Moreover, Angelakos 1964) has recently reported that the NA content of a normal iris increases 500 per cent after incubation *in vitro* in Tyrode's solution containing 50 μg/ml of NA and 1 μg/ml of adenosine triphosphate (ATP).

The present report deals with the histochemically demonstrable NA content of the iris of the rat after *in vitro* incubation in solutions containing NA, dihydroxyphenylalanine (DOPA), tyramine (TA), dopamine (DA), adrenaline (A) or 5-hydroxytryptamine (5-HT).

MATERIAL AND METHODS

Albino rats of the Sprague–Dawley strain were used. The animals were killed by decapitation after a short ether anaesthesia. The iris was removed as a whole and transferred immediately into the incubation medium.

The basic medium consisted of Krebs' salt solution supplemented with calcium chloride (1 ml of 1·22 per cent $CaCl_2$ into 100 ml). This medium was used as such or after addition of 1 mg/ml of glucose and/or 0·001–·0 mg/ml of ATP.

After dissolving in the above media–100 μg/ml of DOPA, TA, DA, NA, A or 5-HT, the pH was adjusted at 7·0. The incubation was carried out at 7°C without agitation. After incubation the iris was washed in three changes of the medium in which no amine was dissolved, for 10 min each. It was then stretched on a slide and allowed to dry for 10 min. The slide was subsequently exposed to dry formaldehyde vapour for 15 min–16 hr at 0–80°C to render the amines into fluorescent compounds, which are formed at different rates from different amines (Eränkö, 1964). The specimens were then mounted in Entellan and examined in the fluorescence microscope.

RESULTS

Incubation in the Krebs' salt solution, with or without glucose or ATP, was followed by a slow weakening in the intensity of the catecholamine fluorescence in the sympathetic nerve net of the iris. No essential difference was observed between specimens exposed directly to formaldehyde and those

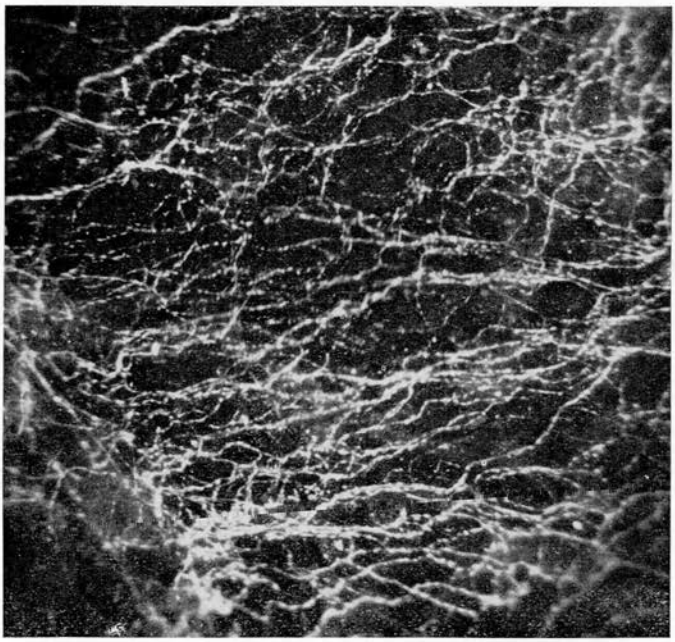


FIG. 1. Formaldehyde-induced fluorescence in an iris incubated for 2 hr in Krebs-glucose solution. The sphincter is in the lower left corner. ×100.

first incubated for 1–2 hr in the Krebs' solution. After 4–5 hr incubation, a slight loss of fluorescence was observed. Specimens incubated overnight in Krebs-glucose still exhibited, though more weakly, the specific fluorescence in the nerve fibres. If glucose was not present, a total loss of fluorescence occurred.

Incubation of the iris with up to 100 μg/ml of NA for 2–4 hr caused but a slight or no increase in the fluorescence intensity of the adrenergic nerve net, whether or not 1 mg/ml of glucose and up to 1 mg/ml of ATP were present.

Incubation in solutions containing 100 μg/ml of DOPA, TA, DA, A or 5-HT caused in 1·5–2 hr a marked decrease in the fluorescence intensity of the nerve fibres (Table 1). While formaldehyde does not render TA fluorescent, the other compounds employed are demonstrable with the present

TABLE 1. INTENSITY OF CATECHOLAMINE FLUORESCENCE IN THE NERVE FIBRES OF THE RAT IRIS INCUBATED FOR 2 HOURS IN KREBS-GLUCOSE WITH DIFFERENT COMPOUNDS (100 μg/ml)

Incubation medium	*Fluorescence intensity*
None (fresh control)	++++
Krebs-glucose (incubation control)	++++
Noradrenaline	+++++
Dihydroxyphenylalanine	+
Tyramine	+
Dopamine	+
Adrenaline	+
5-Hydroxytryptamine	+

technique, although a longer exposure is necessary to convert A into a fluorescent compound. An increase in the fluorescence intensity of the tissues between the nerve fibres was indeed observed, but only after incubation in DOPA or DA, while the nerve fibres had become almost indistinguishable.

The effect of incubation in A is illustrated in Figs. 1 and 2 showing,

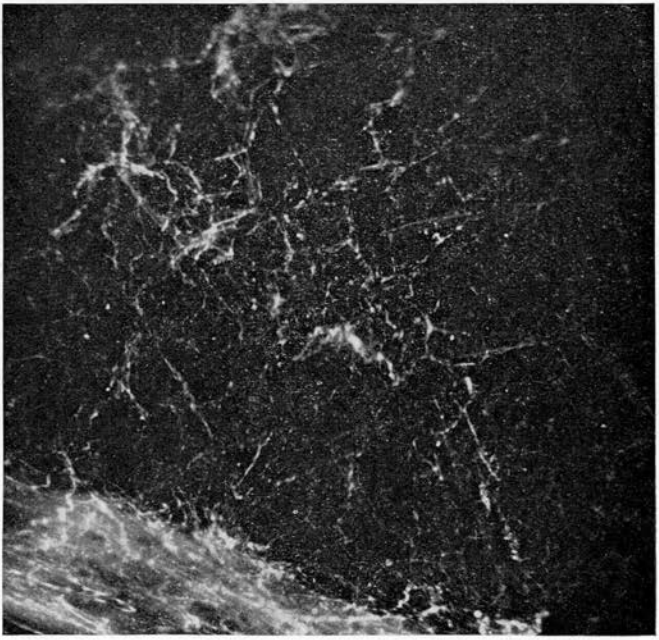


FIG. 2. Formaldehyde-induced fluorescence in an iris incubated for 2 hr in Krebs-glucose solution containing 100 μg/ml. of adrenaline. The reaction is less intense in the dilatator fibres but it is somewhat diffusely increased in the sphincter (lower left corner). $\times$100.

respectively, an iris incubated for 2 hr in Krebs-glucose and another incubated in the same solution in which 100 μg/ml of A was dissolved. There was often an almost total loss of fluorescence. If the iris was incubated in a mixture containing besides 100 μg/ml of A also 10 μg/ml of NA, no loss of fluorescence occurred.

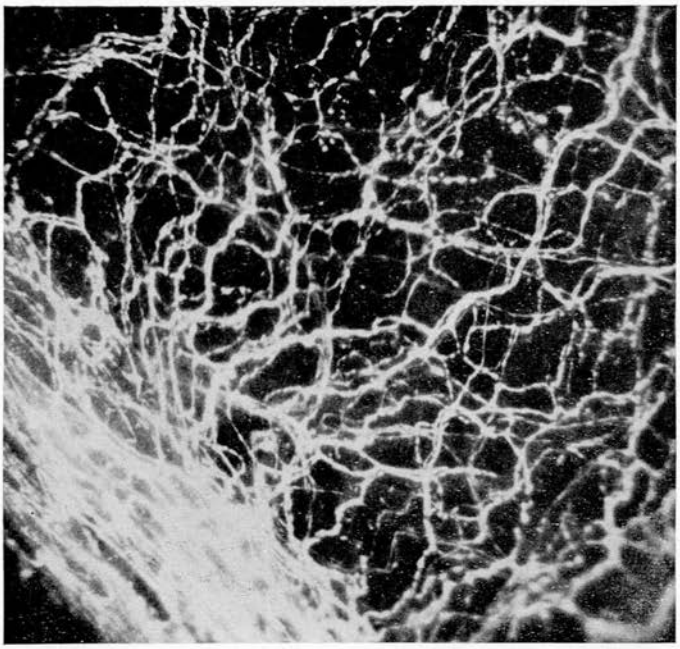


FIG. 3. Formaldehyde-induced fluorescence in an iris first incubated for 2 hr with 100 μg/ml. of adrenaline, washed in Krebs-glucose, and incubated for 2 hr with 100 μg/ml. of noradrenaline. Note the intense fluorescence of all fibres including the preterminal ones and those in the sphincter (lower left corner). $\times$100.

If an iris whose NA stores were thus depleted with A was subsequentl incubated with NA for 2 hr, the adrenergic nerve net became fluorescer again (Fig. 3, Table 2). The intensity of the fluorescence often somewha exceeded that in a control iris exposed to formaldehyde without incubatio A concentration of 1 μg/ml of NA was sufficient to restore the fluorescenc However, the presence of glucose in the incubation medium was necessar to obtain such restitution. Addition of ATP in a concentration of 1 μg 1 mg/ml had no effect.

Besides the terminal fibres, which normally exhibit an intense fluorescenc after exposure to formaldehyde, incubation in NA-containing solutio rendered strongly fluorescent also the smooth preterminal fibres of the iri which normally contain little NA. This occurred equally, whether the ir

TABLE 2. INTENSITY OF CATECHOLAMINE FLUORESCENCE IN THE NERVE FIBRES OF THE RAT IRIS INCUBATED WITH ADRENALINE AND NORADRENALINE (100 μg/ml)

Incubation medium and time	*Fluorescence intensity*
Krebs-glucose 4 hr (control)	++++
Adrenaline 2 hr	+
Adrenaline 4 hr	±
Adrenaline 2 hr, Noradrenaline 2 hr	+++++
Noradrenaline 4 hr	+++++

was first exposed to A or some other amine and thereafter to NA, or whether it was directly incubated with NA.

DISCUSSION

Our results supplement the *in vivo* experiments carried out by Hillarp and Malmfors (1964), who observed that exogenous administration restores the NA content of the nerve fibres of the iris, if these were first depleted with reserpine. On the other hand, our observations are at variance with those made by Angelakos (1964), who found a sevenfold increase in the NA concentration of the normal iris after incubation *in vitro* with NA, ATP being necessary to obtain this effect. This discrepancy is difficult to explain but it is possible that Angelakos employed longer incubation times (not given in his paper).

Observations of the present study indicate that DOPA, TA, DA, A and 5-HT are capable of releasing NA from the adrenergic fibres of the iris, apparently without occupying the binding sites of NA permanently. Since the loss of fluorescence was marked also in the normally intensely fluorescent varicosities of the nerve net, in which NA is probably bound in granules, these compounds must affect both the axonal and the granular pool of NA.

Why DOPA, TA, DA, A or 5-HT did not become attached to the nerve fibres is an interesting problem. One explanation is that NA is chemically bound in many different ways (Stjärne, 1964). Musacchio *et al.* (1965) proposed that the amino group, the catechol hydroxyl group and the β-hydroxyl group all play a part in binding amines in sympathetic nerves. Of the compounds employed in the present study, only NA possesses all of these three groups, except for A, whose amino group is methylated. Whatever the mode of binding may be, the experiments described demonstrate its high specificity to NA.

Finally, it seems to be of considerable physiological interest that amines such as DA, A and 5-HT are capable of releasing NA largely without being bound in its binding sites in the sympathetic nerve terminals.

SUMMARY

Incubation *in vitro* of rat's iris in Krebs' salt solution containing dihydroxyphenylalanine, tyramine, dopamine, adrenaline or 5-hydroxytryptamine caused a dramatic loss of histochemically demonstrable noradrenaline from the sympathetic nerve net of the rat iris. Incubation of normal iris with noradrenaline had little effect on the noradrenaline content of the fibres but restored normal histochemical reaction in iris whose noradrenaline was first depleted with one of the above compounds. Glucose was necessary for such restoration but adenosine triphosphate had no effect on it.

REFERENCES

ANGELAKOS, E. T. (1964) Histochemical demonstration of uptake of exogenous norepinephrine by adrenergic fibres *in vitro*, *Science* **145**, 503–4.

ERÄNKÖ, O. (1964) Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour, *J. Histochem. Cytochem.* **12**, 487–8.

HILLARP, N.-Å. and T. MALMFORS (1964) Reserpine and cocaine blocking of the uptake and storage mechanisms in adrenergic nerves, *Life Sci.* **3**, 703–8.

MUSACCHIO, J. M., V. K. WEISE and I. J. KOPIN (1965) Mechanism of norepinephrine binding, *Nature* (*Lond.*) **205**, 606–7.

STJÄRNE, L. (1964) Studies of catecholamine uptake, storage and release mechanisms, *Acta Physiol. Scand.* **62**, Suppl. 228.

DISCUSSION

HAMBERGER: I would like to comment on the depletion of noradrenaline by dopamine in rat iris incubated *in vitro*. In extensive *in vitro* and *in vivo* experiments with dopamine, other catecholamines and DOPA we have in Hillarp's laboratory never found any signs of lowering of the catecholamine fluorescence. On the contrary, we have found that the whole adrenergic neuron has a very efficient uptake-concentration mechanism demonstrable at low concentrations (*in vitro* down to 0·01 ug/ml), located to the level of the cell membrane and insensitive to reserpine.

WEINER: Angelakos (1964) has shown, both by fluorimetric and histochemical studies that norepinephrine is taken up and concentrated in the iris *in vitro*, presumably in nerve terminals, when ATP is added to the incubation medium. Perhaps you are not obtaining increased fluorescence because of an absence of energy for this uptake mechanism. Have you correlated your studies with biochemical values?

ERÄNKÖ: I am glad to hear Dr. Hamberger's comment, which shows that our observations are new. The loss of catecholamine fluorescence due to incubation in the amines which we employed and to subsequent washing in Krebs-glucose to remove any loosely bound amines is readily reproducible. An iris depleted in such a mild way of itw noradrenaline stores seems a useful preparation for the study of the uptake of catecholamines by sympathetic nerves.

To Dr. Weiner I should like to say that we also observed, like Angelakos, an increase in the fluorescence intensity after incubation with noradrenaline, although a smaller one and not dependent on the presence or absence of ATP. We did not make quantitative determinations with biochemical methods.

Acta physiol. scand. 1966. 66. 253—254

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Finland

Adrenaline and Noradrenaline in the Organ of Zuckerkandl and Adrenals of Newborn Rats Treated with Hydrocortisone

By

OLAVI ERÄNKÖ, MATTI LEMPINEN and LIISA RÄISÄNEN

Adrenals of newborn rats contain noradrenaline (NA) and adrenaline (A) in the same proportion, about 1:5, as adrenals of adult rats (Eränkö and Räisänen 1957). No such information is available of the extra-adrenal chromaffin tissue of the rat, although histochemical observations show that it contains catecholamines at birth but degenerates in about a week thereafter, unless adrenocortical hormone is given daily (Lempinen 1964). Treatment with hydrocortisone results in an increase in the intensity of the chromaffin reaction but in a loss of formalin-induced fluorescence (Lempinen 1965).

In the present study, the main para-aortic chromaffin body, *i.e.* the Organ of Zuckerkandl (OZ), and the adrenals were examined for NA and A in normal and hydrocortisone-treated young rats.

Newborn albino rats were injected daily with 0.1 mg of hydrocortisone for 7 days. Since the extra-adrenal chromaffin tissue has degenerated in normal 7-days-old rats, normal newborn rats were used as controls. The animals were killed by decapitation and the retroperitoneal tissue block containing the OZ was frozen and sectioned serially. The OZ and the adrenals were dissected from frozen dried sections. Material thus collected from 6 animals was pooled and applied to the base line of the chromatography paper. Small pieces of frozen dried adrenal medulla of adult rats served as controls. The chromatogram was developed with phenol / 0.1 N hydrochloric acid and the catecholamines were made fluorescent by spraying with 2 % potassium ferricyanide in phosphate buffer at pH 7.1.

A typical chromatogram is shown in Fig. 1. The OZ of the newborn control rats (Z) contained NA only, while both NA and A were found in the OZ of hydrocortisone-treated rats (Z'), the NA / A proportion being about the same as that in the adult adrenal medulla (unmarked). While hydrocortisone thus radically affected the catecholamine composition of the OZ, it had apparently no such effect on the adrenal medulla, which contained both NA and A in newborn controls (A) and in hydrocortisone-treated (A') rats.

The presented observations provide for the first time direct evidence of the effect of a cortical hormone on the methylation of catecholamines in chromaffin cells, which has been earlier suspected on a speculative basis (see review in Lempinen 1963). However, it is not

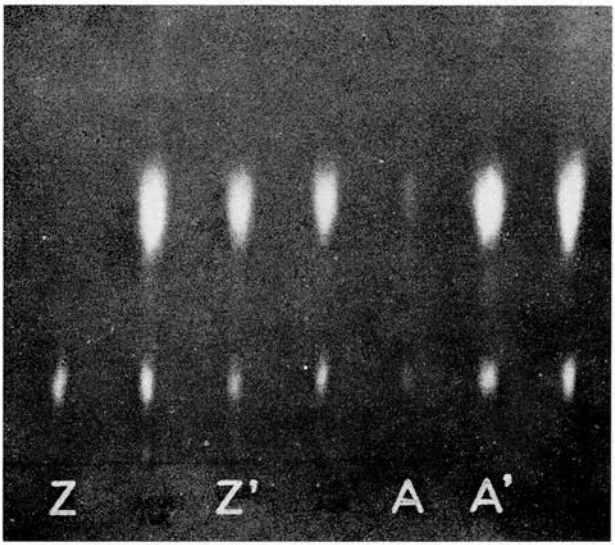


Fig. 1. Fluorescence photograph of a chromatogram developed in phenol-hydrochloric acid for 7 hours. The upper spots are due to adrenaline, the lower ones to noradrenaline. Z, the organs of Zuckerkandl of 6 newborn rats pooled together; Z', the same organs of 6 rats injected with hydrocortisone for 7 days. A, adrenals of 6 newborn rats; A', adrenals of 6 injected rats. The unmarked spots are obtained from adult adrenal medulla.

possible to say with certainty whether the appearance of A is directly due to hydrocortisone itself or to other factors which have the chance to become operative when the normal degeneration of the extra-adrenal chromaffin cells is prevented with the aid of hydrocortisone.

References

ERÄNKÖ, O., and L. RÄISÄNEN, Adrenaline and noradrenaline in the adrenal medulla during postnatal development of the rat. *Endocrinology* 1957. *60*. 753—760.

LEMPINEN, M., Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. *Acta physiol. scand.* 1964. Suppl. 231.

LEMPINEN, M., Effect of hydrocortisone on histochemically demonstrable catecholamines of the para-aortic body of the rat. *Acta physiol. scand.* 1966. *66*. 251—252.

KUNGL. BOKTR. STHLM 1966

PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

Printed in U.S.A

G. DEMONSTRATION OF CATECHOLAMINES AND CHOLINESTERASES IN THE SAME SECTION

OLAVI ERÄNKÖ

Department of Anatomy, University of Helsinki

Catecholamines form intensely fluorescent compounds when treated with formaldehyde. A method for the histochemical demonstration of norepinephrine (NE) in the adrenal medulla based on this principle was reported over 10 years ago (1, 3). In this method, fresh tissue was fixed in an aqueous solution of formaldehyde and sections from it were examined in the fluorescence microscope; the specificity of the method was controlled by direct chemical analysis of microdissected pieces of tissue (3).

Lagunoff *et al.* (20) were the first to employ formaldehyde in vapor form for histochemical localization of monoamines. About the same time, the high sensitivity of the formaldehyde vapor technique was observed by Falck and Torp (12) and by the present writer (5–7). Application of this method to nervous tissues (11) has since then proved fruitful in the microscopic study of monoaminergic neurones, as will be evident from other papers of the present symposium.

While the above techniques make it possible to localize catecholamines in tissue sections, histochemical demonstration of other transmitter substances is not yet possible. However, it is possible to demonstrate histochemically an enzyme closely associated with cholinergic transmission, *i.e.*, acetylcholinesterase (16, 19). Studies carried out with this method have provided valuable information, and have led to a new hypothesis concerning cholinergic transmission (17, 18).

In view of the close interaction between monoaminergic and cholinergic systems it appeared of interest to try to demonstrate in the same tissue section both catecholamines and cholinesterases. If an aqueous solution of formaldehyde is used for the localization of NE, which is possible only in organs with high concentrations of firmly bound catecholamine, such as the adrenal medulla, little difficulty is encountered in subsequent demonstration of cholinesterases. It was thus found, for example, that a nerve net with a high activity of nonspecific cholinesterase is selectively associated with the NE-containing cell groups of the adrenal medulla, while the fine fibers exhibiting acetylcholinesterase activity were evenly distributed through the whole adrenal medulla (4).

Combination of the formaldehyde vapor technique with the acetylcholinesterase method obviously provides possibilities with a wider scope in the correlative study of aminergic and cholinergic structures. Unfortunately, formaldehyde vapor destroys cholinesterase activity much more strongly than does formaldehyde solution. Great care is therefore necessary to find an exposure to formaldehyde sufficient to bring about the amine fluorescence but still preserving enough acetylcholinesterase activity to make possible its subsequent demonstration.

Such a technique, a brief report of which has been published (6), has been applied to sympathetic nervous structures of the rat (8–10, 14). It consists of four main steps: 1) freeze-drying; 2) exposure to formaldehyde; 3) fluorescence microscopy; 4) demonstration of cholinesterases. Since the margin of safety is narrow, all of these steps are critical. Although it is not possible to go into all details in the present paper, some essential features of the method will be given.

1. FREEZE-DRYING

Rapid freezing is necessary to avoid ice crystal artifacts and displacement of the amines. Tissue slices less than about 0.5 mm in thickness are placed on copper foils and cooled either with copper disc forceps previously immersed in liquid air (2) or by direct immersion in liquid air or cooled isopentane. The cold tissue is then dried in any good freeze-drying apparatus (see 21). The temperature *of the tissue* must be lower than −40°C. To avoid overly long drying periods the temperature of the tissue holder should be thermostatically controlled, an oil diffusion pump should be used, and a trap cooled with liquid air should be provided close to the tissue holder. With an efficient apparatus the drying time can be reduced to 1 to 2 days.

2. EXPOSURE TO FORMALDEHYDE

After drying, the temperature is increased to +40°C, the vacuum is broken, and the piece of tissue is either directly embedded in paraffin wax under vacuum or before embedding exposed as such to formaldehyde vapor. The former method makes it possible to treat sections individually with formaldehyde, which is helpful in finding out the optimal exposure conditions.

If the relative humidity of the air exceeds 40 %, special precautions are necessary to avoid absorption of water vapor by the dried tissue, with resulting displacement of the water-soluble amines. Plastic chambers kept dry with silica gel are essential for storing the tissue both before and after exposure to formaldehyde.

The exposure conditions vary with the nature of the tissue, the size of the piece, the temperature of exposure, the ambient humidity, and the water content of the paraformaldehyde. Accordingly, the optimal conditions must be found by trial and error. We have obtained good results by exposing whole superior cervical ganglia of the rat to formaldehyde gas released from paraformaldehyde at 50°C for 30 min. The paraformaldehyde was first equilibrated with air whose relative humidity was 40 %. Paraformaldehyde was then poured onto the bottom of a Petri dish 15 cm in diameter, the warm tissue holder was placed in the center, and the dish was closed and transferred into an incubating oven at 50°C.

After formaldehyde treatment the tissue pieces are embedded in paraffin wax under vacuum and sectioned. Fluorescence microscopy can be done without deparaffinization by melting the paraffin wax; but deparaffinization with, and mounting in, xylene is usually desirable, although some of the fluorescent material, notably epinephrine (E), may be lost.

3. FLUORESCENCE MICROSCOPY

Ultraviolet light has an inactivating effect on cholinesterases. Exposure to it must therefore be kept to a minimum, and this means almost immediate photography. A microscope lamp incorporating a Siemens HBO 200 mercury burner, Schott filters BG 12 and OG 1, a dark field condenser, nonfluorescent glass optics, and any film with an exposure index greater than ASA 200 are sufficient to provide photographic exposures shorter than 30 sec. During exposure, the coordinates of the slide on the microscope stage should be registered for ater relocation.

4. DEMONSTRATION OF CHOLINESTERASES

Immediately after fluorescence microscopy the tissue sections are transferred into the incubation mixture. Acetylthiocholine and butyrylthiocholine are recommended as substrates, the former in combination with 10^{-6} M iso-OMPA (tetra-isopropylpyrophosphoramide) for the selective demonstration of acetylcholinesterase, the latter together with 10^{-5} M B. W. 284 C 51 [1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide] for the selective demonstration of nonspecific cholinesterases (see 21). Successful results can be obtained with Koelle's improved technique (16), with Gomori's simplified version (13), and with the recent modification by Karnovsky and Roots (15). The incubation period should be long enough (often more than 4 hr) to detect the activity weakened after treatment by formaldehyde. The use of the above inhibitors or others more selective than DFP (di-isopropyl fluorophosphonate) is strongly recommended to obtain proper discrimination between acetylcholinesterase and nonspecific cholinesterase (see 21).

With care, satisfactory results can be obtained. Figure 1a shows the distribution of NE in the superior cervical ganglion of the rat. Figure 1b is the same field after demonstration of acetylcholinesterase activity. Identification of the same individual cells in both photomicrographs is readily possible, and it can be seen that intense acetylcholinesterase activity can be associated with a weak, moderate, or strong fluorescence, and *vice versa*. Cells with an intense fluorescence and an intense enzyme activity are of special interest in view of the hypothesis of Burn and Rand postulating both adrenergic and cholinergic transmission mechanisms in the same neurone (see 18).

Figure 2a shows formaldehyde-induced fluorescence in the rat iris. Acetylcholinesterase activity of the same field is shown in figure 2b. Correlation of the fluorescence and the enzyme reaction in the individual fibers is difficult when the pictures are side by side, because the fluorescent fibers exhibit a pattern essentially different from that of the cholinesterase positive fibers. Spatial correlation is facilitated by examination of superimposed prints over a strong light. It can thus be seen that many of the fine, strongly fluorescent fibers with synaptic enlargements are exactly in the same site as some of the cholinesterase positive fibers. While other interpretations are possible, it is attractive to speculate that such fibers, which are far fewer than fibers which are *either* fluorescent *or* cho-

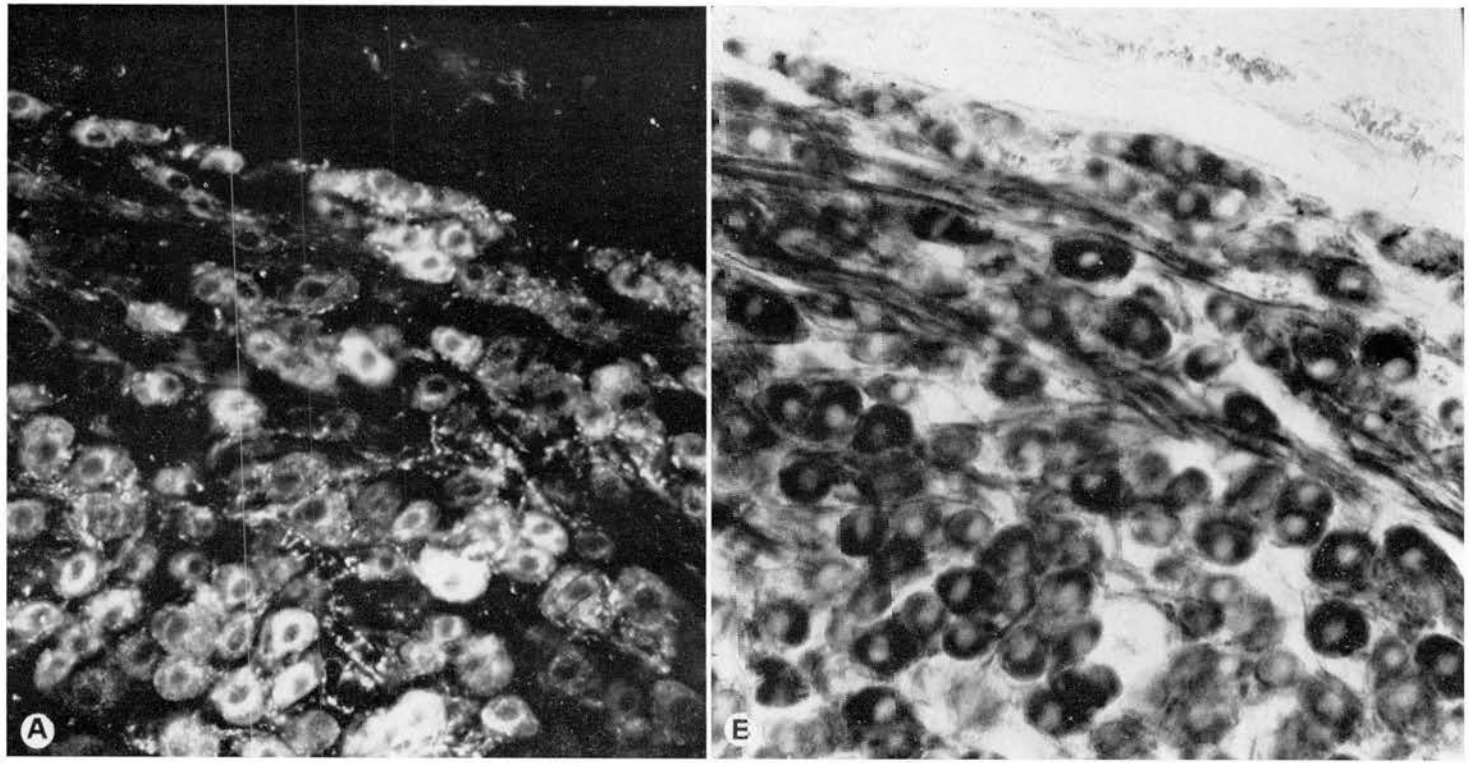


FIG. 1a. Superior cervical ganglion of the rat. Fluorescence photomicrograph of tissue exposed to formaldehyde vapor at 50°C for 30 min.
FIG. 1b. The same field as in figure 1a after demonstration of acetylcholinesterase activity.

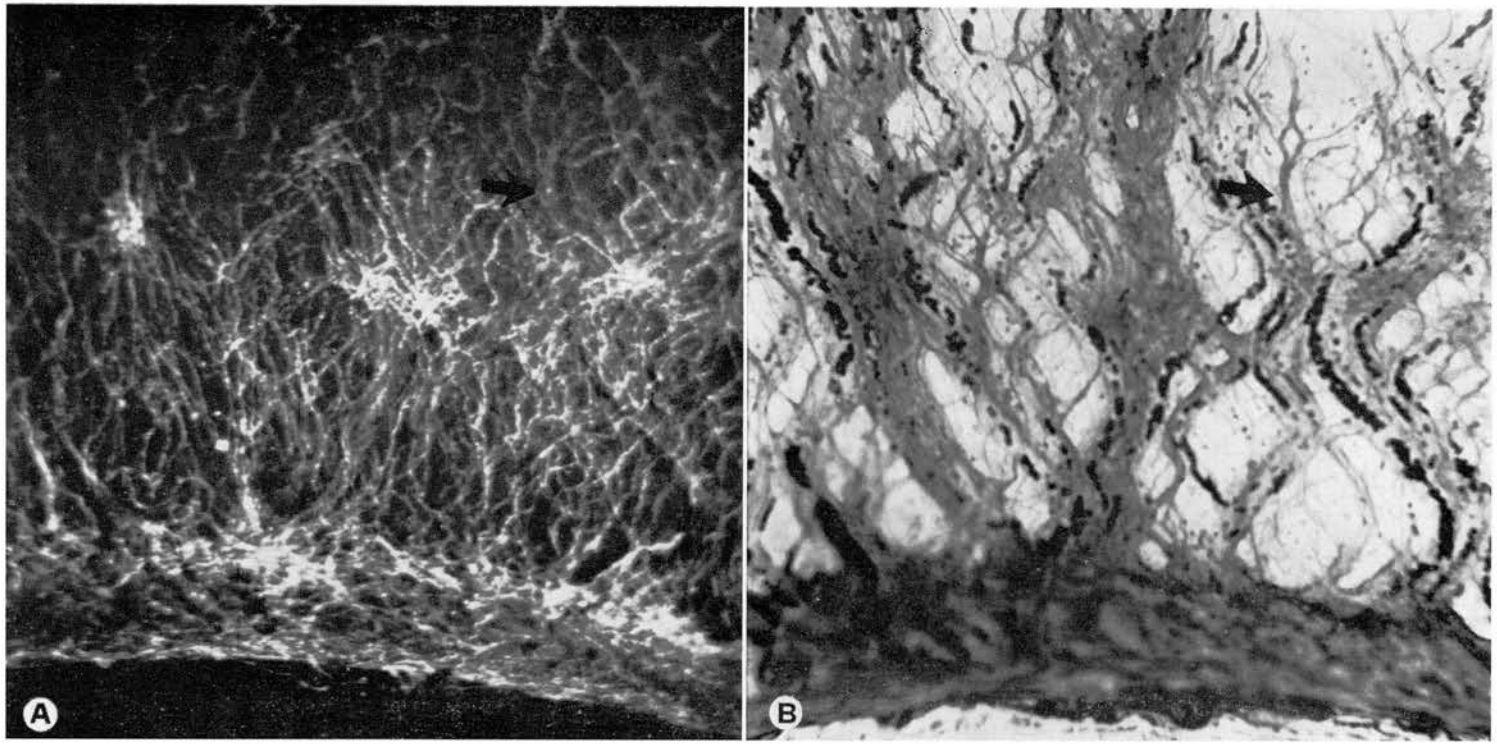


FIG. 2a. Whole mount of the rat iris. Fluorescence photomicrograph obtained after the tissue was exposed to formaldehyde vapor for 20 min at 40°C.

FIG. 2b. Same field as in figure 2a after demonstration of acetylcholinesterase activity. The pattern is different but fine fibers with the same course as that of the fluorescent fibers can be found (arrow). The black rows of dots are red blood cells.

linesterase positive ones, originate from those cells of the superior cervical ganglion which were shown to contain both an intense fluorescence and an intense acetylcholinesterase activity.

These examples may suffice to indicate the potential value of the combination technique. Its application to the study of individual synaptic boutons appears especially interesting, and work is in progress to study this problem.

REFERENCES

1. ERÄNKÖ, O.: On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. Acta anat. **16:** suppl. 17, 1952.
2. ERÄNKÖ, O.: Quenching of tissues for freeze-drying. Acta anat. **22:** 331–336, 1954.
3. ERÄNKÖ, O.: Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. Acta endocr., Copenhagen, **18:** 174–179, 1955.
4. ERÄNKÖ, O.: Specific demonstration of acetylcholinesterase and non-specific cholinesterase in the adrenal gland of the rat. Histochemie **1:** 257–267, 1959.
5. ERÄNKÖ, O.: Personal communication to Dr. Falck acknowledged by Falck and Torp (loc. cit.), 1961.
6. ERÄNKÖ, O.: Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. J. Histochem. Cytochem. **12:** 487–488, 1964.
7. ERÄNKÖ, O. AND HÄRKÖNEN, M.: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. Acta physiol. scand. **58:** 285–286, 1963.
8. ERÄNKÖ, O. AND HÄRKÖNEN, M.: Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. Acta physiol. scand. **61:** 299–300, 1964.
9. ERÄNKÖ, O. AND HÄRKÖNEN, M.: Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. Acta physiol. scand. **63:** 411–412, 1965.
10. ERÄNKÖ, O. AND RÄISÄNEN, L.: Fibres containing both noradrenaline and acetylcholinesterase in the nerve net of the rat iris. Acta physiol. scand. **63:** 505–506, 1965.
11. FALCK, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. **56:** suppl. 197, 1962.
12. FALCK, B. AND TORP, A.: A fluorescence method for histochemical demonstration of noradrenalin in the adrenal medulla. Med. exp. **5:** 429–432, 1961.
13. GOMORI, G.: Microscopic Histochemistry, Principles and Practice, The University of Chicago Press, Chicago, 1952.
14. HÄRKÖNEN, M.: Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. Acta physiol. scand. **63:** suppl. 237, 1964.
15. KARNOVSKY, M. J. AND ROOTS, L.: A "direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. **12:** 219–221, 1964.
16. KOELLE, G. B.: The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. J. Pharmacol. **103:** 153–171, 1951.
17. KOELLE, G. B.: A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. J. Pharm., Lond. **14:** 65–90, 1962.
18. KOELLE, G. B.: Cytological distributions and physiological functions of cholinesterases. In: Handb. exp. Pharm., Ergänzungswerk, vol. 15, pp. 187–298, Springer-Verlag, Berlin, 1963.
19. KOELLE, G. B. AND FRIEDENWALD, J. S.: A histochemical method for localizing cholinesterase activity. Proc. Soc. exp. Biol. N.Y. **70:** 617–622, 1949.
20. LAGUNOFF, D., PHILLIPS, M. AND BENDITT, E. V.: The histochemical demonstration of histamine in mast cells. J. Histochem. Cytochem. **9:** 534–541, 1961.
21. PEARSE, A. G. E.: Histochemistry Theoretical and Applied. J & A. Churchill Ltd., London, 1960.

THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 14, No. 9

Printed in U.S.A.

Letters to the Editor

SOME BRITISH COMMENTS ON THE HISTOCHEMICAL NOMENCLATURE OF MUCOSUBSTANCES

Inasmuch as our views are typical of British histochemists and biochemists, we wish to make plain our attitude toward the system of histochemical nomenclature of mucosubstances proposed by Spicer *et al.* (*J. Histochem. Cytochem., 13:* 599. 1965).

We affirm the view of Spicer *et al.* that histochemists should, in histochemical publications, use the systematic and trivial names coined by biochemists *provided* that these names are used in a proper context and wherever they are relevant. But we fail to see how histochemists can unequivocally identify a particular mucosubstance (or even its class, for example, glycoprotein) in a particular tissue site with the histochemical methods *currently* available. Nevertheless, to be fair, we recognize that histochemists have to face problems in this field that are harder in many respects than those which biochemists usually have to cope with.

Therefore, we favor the scheme put forward by Spicer *et al.* of describing mucosubstances for histochemical purposes in terms of the locating reagents used under rigorously defined conditions. On the other hand, histochemists might be wiser to exercise greater caution about interpreting the results of such tests in terms of chemical structure than they sometimes do at present, although they should take comfort in knowing that even biochemists cannot always say confidently what they mean by the terms they employ.

There are a few matters of detail that could be criticized about the Spicer *et al.* scheme. Some of these have been fairly and adequately dealt with by Meyer (*J. Histochem. Cytochem. 14:* 605. 1966) The term 'periodate-reactive' is a case in point. Here, as long as biochemists know that 'periodate-reactive' means to histochemists a mucosubstance which reacts with carbonyl-reactive reagents (for example, Schiff's reagent) after *10-min* oxidation with periodic acid, no confusion should occur. The term should not imply to a histochemist that if a mucosubstance does not, for example, give a positive periodic acid-Schiff reaction, then the mucosubstance contains no *vic*-glycol groups; biochemists could argue that the mucosubstance may not have been oxidized for long enough.

In conclusion, the Spicer *et al.* scheme of terminology seems to us to be a useful and timely one for histochemical purposes, especially as it is intelligible to biochemists. Of course, its details will change, but its basic structure should be useful for some time yet. The only real way of resolving the difficulties between the two disciplines is for histochemists and biochemists to move more closely together and to tackle jointly the development of methods of locating, separating and identifying the complex carbohydrates present in subcellular particles and particular types of cells.

July 13, 1966

P. J. STOWARD
Department of Human Anatomy
University of Oxford
Oxford, England
S. A. BARKER
Department of Chemistry
University of Birmingham
Birmingham, England
P. W. KENT
Department of Biochemistry
University of Oxford
Oxford, England
A. G. E. PEARSE
Department of Histochemistry
Postgraduate Medical School
London, England

SOME COMMENTS ON THE MECHANISM OF THE SCHIFF REACTION

In a paper in this *Journal* (*12:* 748. 1964), Hardonk and van Duijn concluded that the products formed between Schiff's reagent and aldehydes in model film systems are alkyl sulfonic acids rather than the *N*-sulfinic acids which had been favored by previous reviewers of the Schiff reaction (*e.g.*, Kasten, *Int. Rev. Cytol. 10:* 1. 1960). Nauman *et al.* (*Anal. Chem. 32:* 1307. 1960) had earlier reached a similar conclusion from *in vitro* studies.

According to Nauman *et al.*, the main absorption bands of aqueous solutions of pararosaniline (the principal component of Schiff's reagent) are

Present address: Department of Human Anatomy, South Parks Road, Oxford, England.

located at 538, 280 and 230 mμ. The band at 538 mμ is typical of the lowest energy band of all triphenylmethane dyes and is associated with the molecule as a whole in the form of a positive (electron-deficient) ion which allows the electrons on the nitrogen atoms to interact through the central carbon atom. The visible band is, therefore, eliminated if the central methane carbon atom in the molecule is bonded to chloride or sulfites for example. Any shifts in this band are most probably due to solvent effects (*e.g.*, λ max is 548 mμ in acetone but is 543 mμ in benzyl alcohol). Hardonk and van Duijn found that the visible absorption spectra of synthetic aldehyde films stained with (a) pararosaniline, or with (b) pararosaniline followed by a sulfite rinse or with (c) Schiff's reagent are identical in shape with maxima 7 mμ longer than that of a solution of pararosaniline in benzyl alcohol; from this they conclude that, as the reaction product in (b) cannot contain an *N*-sulfinic acid, it is probably a sulfonic acid, and therefore the reaction product in (c) is also probably a sulfonic acid. In my view, their data can be reinterpreted in such a way that it is not possible to decide whether the final product in experiment (c) is an alkyl sulfonic acid, an *N*-sulfinic acid or even an azomethine Schiff base.

Considering for the sake of simplicity only monosubstitution products, two canonicals of the resonance structure of the reaction product (an azomethine Schiff base) formed in experiment (a) are Ia and Ib (Fig. 1). This structure contains an electrophilic center on the carbon atom of the azomethine bond. Therefore, if this reaction product is treated with bisulfite in the form of sulfurous acid (as in experiment b), it is highly probable that a compound with a resonance canonical II would be formed.

For the reaction between aldehyde films and Schiff's reagent (experiment c), Nauman *et al.* would argue that, on the alkyl sulfonic acid theory, the aldehyde first reacts with the sulfur dioxide present in Schiff's reagent to form an alkyl α-hydroxy sulfonic acid intermediate which immediately reacts with the colorless anilinium-like pararosanaline ion to give a colored product III, one of whose resonance canonicals would be the same as II.

On the other hand, if the *N*-sulfinic acid theory is the operative one for experiment (c), the canonicals corresponding to II and III would be IVb (with a smaller contribution to the resonance structure from IVa) and V respectively. All these canonicals contain the same chromophore (*i.e.*, the electron-deficient central methane carbon atom) giving rise to the 538-mμ adsorption band, and therefore it cannot be inferred *from this band alone* which resonance structure (II or V) can be

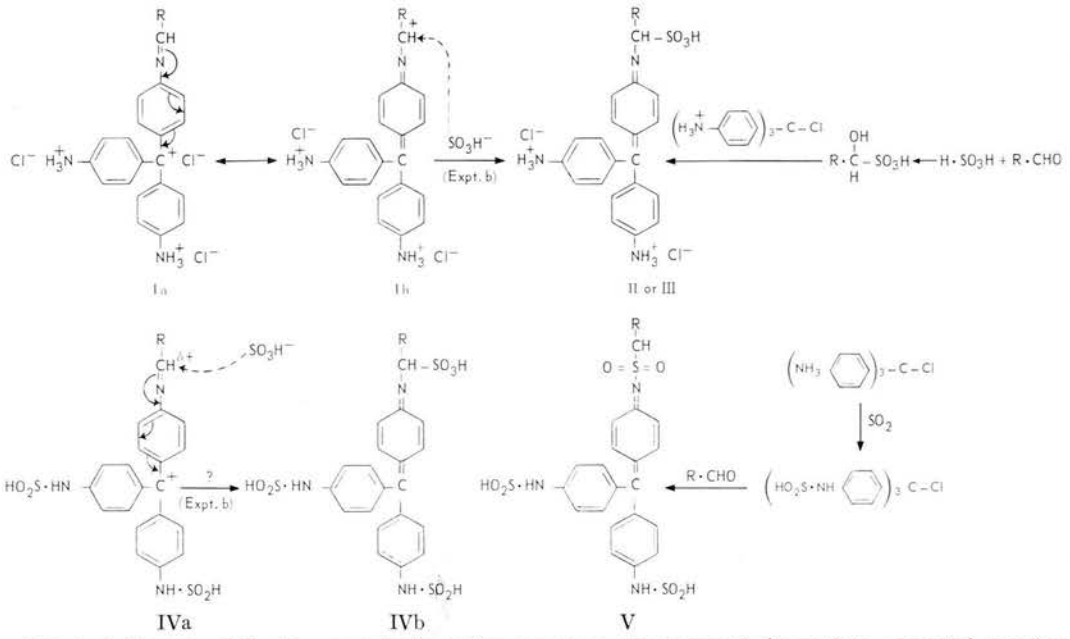


FIG. 1. I–V are contributing canonicals to the resonance structures of the various potential reaction products formed in Hardonk and van Duijn's experiments as described in the text. The two reaction schemes drawn on the right summarize the mechanism of the Schiff-aldehyde reaction according to the alkyl sulfonic acid (final product III) and *N*-sulfinic acid (final product V) theories respectively.

assigned to the Schiff reaction product in experiment (c).

Although the sequence of adding reagents in experiment (b) precludes the formation of *N*-sulfinic acids in that experiment, I respectfully disagree with Hardonk and van Duijn that because the visible absorption spectrum obtained in experiment (b) is the same as that obtained in experiment (c), it *necessarily* implies that the products obtained in these two experiments are the same. That the spectra are identical could be entirely fortuitous. However, I agree with Hardonk and van Duijn that the balance of evidence adduced for mechanisms of the Schiff reaction lies in favor of the sulfonic acid theory. The results of their experiments and the earlier work of Nauman *et al.* bear this conclusion out.

A cytophotometric analysis of stained aldehyde films in the 280- and 232- mμ regions, together with the effect of pH on the maxima, might provide the much needed information about the nature of the aldehyde-sulfur dioxide-amine linkage and its effect on the formation of visibly colored structures involving (in the classical chemical sense) the loss of hydroxyl, chloride or sulfite from the central methane carbon atom of the dye. This is not as easy as it might at first appear. In Hardonk and van Duijn's system, for example, the deoxyribonucleic acid pyrimidine absorption bands may obscure any changes in the ultraviolet absorption peaks originating from the aldehyde-Schiff dye linkage.

I am indebted to Drs. Hardonk and van Duijn for their most helpful views on the first draft of this communication.

June 10, 1966

P. J. Stoward
Laboratory of Experimental Pathology
National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda, Maryland 20014

REPLY TO STOWARD'S LETTER

Stoward's comments on the conclusions that we have drawn from our experimental findings discuss a number of theoretical possibilities, as found in the current literature.

One possibility, namely, that the final product of experiment (c) could be a simple azomethine Schiff base, can, in our opinion, be excluded. It conflicts with our finding that the product of reaction (a) is released in weak acid from the apurinic acid more easily than the product of reaction c and thus apparently has a different structure.

In addition to the evidence given in our papers, we would like to cite the following arguments why we consider the alkylsulfonic acid structure the more likely one: (1) *N*-sulfinic acids are reported by Rumpf (*Ann. Chim. (Paris) 3:* 327. 1935), and (2) the formation of six products in the reaction of the Schiff reagent with formaldehyde (Barka and Ornstein, *J. Histochem. Cytochem. 8:* 286. 1960) is difficult to explain with the sulfinic acid theory.

In answer to Stoward's main argument, the reason that we strongly prefer the alkylsulfonic acid structure for reaction (c), also, is a matter of the application of the principle of Occam's razor. Since, in the case of (b) the alkylsulfonic acid structure is considered likely because of the sequence in which reactants are added, we feel that the simplest explanation is to accept this same structure for the product of reaction (c) in which sulfite and pararosaniline are added together.

Study of the ultraviolet spectra, as proposed by Stoward, would probably yield more information on the identity, or nonidentity, of products (b) and (c).

June 29, 1966

P. van Duijn
Laboratory of Pathology
University of Leiden
Leiden, The Netherlands
M. J. Hardonk
Laboratory of Pathology
University of Groningen
Groningen, The Netherlands

HETEROGENEITY OF NICOTINAMIDE ADENINE DINUCLEOTIDE DIAPHORASE[1]

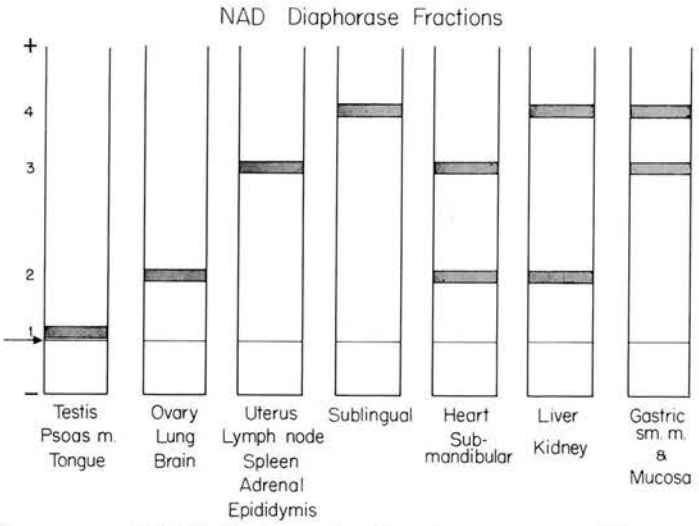


FIG. 1. Zymograms of NAD diaphorase fractions from organs and tissues of the rat.

Several enzymes, including lactate dehydrogenase (LDH), have been demonstrated to exist as multimolecular forms (isozymes) which are separable by electrophoretic and chromatographic procedures (see *Ann. N. Y. Acad. Sci. 94:* 655. 1961). The basis for the heterogeneity of the LDH isozymes has been explained by the work of Markert (In *Hereditary, Developmental and Immunologic Aspects of Kidney Disease*, edited by J. Metcalf, Northwestern University Press, Evanston, Ill., 1962). Two genes (A and B) control the synthesis of two polypeptide subunits (A and B). Assembly of the A and B subunits into tetrameres results in the production of five LDH isozymes (A^4, A^3B^1, A^2B^2, A^1B^3, B^4).

Another heterogenous enzyme, malate dehydrogenase (MDH), has been found by Conklin and Nebel (*J. Histochem. Cytochem. 13:* 510. 1965) to have still another basis for its heterogeneity. In this instance, three genetically unrelated proteins with the same substrate activity are localized in specific cellular organelles. The proteins are genetically unrelated in that, unlike LDH isozymes, they can occur independently of one another.

Previous study (Dewey and Conklin, *Proc. Soc. Exp. Biol. Med. 105:* 492. 1961) indicated that nicotinamide adenine dinucleotide (NAD) diaphorase existed in a heterogenous form in rat kidney. There have been few studies of heterogeneity of components of the respiratory chain beyond the level of the dehydrogenases and therefore we have extended our observations of the isozymes of NAD diaphorase.

In this study, the NAD diaphorases of homogenates of 18 tissues and organs of the rat were separated by gel electrophoresis as previously reported (Dewey and Conklin, *op. cit.*). The substrate employed was that previously utilized for cytochemical demonstration of NAD diaphorase (Conklin, Dewey and Kahn, *Amer. J. Anat. 110:* 19. 1962).

Four electrophoretically different NAD diaphorases were demonstrated in various tissues and organs of the rat (Fig. 1). Twelve of the organs and tissues contained but one of the four diaphorase fractions. In the remaining six organs, two fractions were found and occurred in the combination of fractions 2 and 3, 3 and 4, 2 and 4. Note that diaphorase fraction 1 did not occur in combination with any of the other fractions. There is no obvious relationship between the enzyme patterns (zymograms) of the NAD diaphorases and the LDH isozymes of the rat (Buta, Conklin and Dewey, *J. Histochem. Cytochem. 14:* 658. 1966). There are similarities in the number and occurrence of isozymes of MDH and NAD diaphorase in the liver, kidney and heart of the rat (unpublished observation).

While fragmentation of the respiratory chain (Dewey and Conklin, *op. cit.*) could account for the occurrence of multiple diaphorases, it seems unlikely because of the variation in the zymograms

[1] Supported by Grants AM05197 and HD00557, National Institutes of Health.

of the different organs. It also seems unlikely that the observed zymograms are the result of monomer assortment as in the occurrence of LDH isozymes (Markert, *op. cit.*) for the following reasons: (1) single diaphorase fractions were found with a much greater frequency than would be predicted; (2) no more than two diaphorase fractions occurred in any given organ or tissue; (3) the diaphorase fractions occurred in combinations which are not possible through random assortment of subunits.

The similarity between the diaphorase zymograms and the zymograms of MDH of the chick (Conklin and Nebel, *op. cit.*) and rat suggests a common casual relationship. Each of the diaphorases is synthesized independently of the others and has in common only the ability to act upon the same substrate. In addition, it is likely that specific NAD diaphorase fractions are associated with specific intracellular compartments. The demonstration of diaphorase isozymes of cell fractions from the various organs should provide confirmation of this conclusion.

May 27, 1966

JAMES L. CONKLIN
Department of Anatomy
The University of Michigan
Ann Arbor, Michigan 48104
MAYNARD M. DEWEY
Department of Anatomy
Women's Medical College
Philadelphia, Pennsylvania

HISTOCHEMICAL URATE OXIDASE ACTIVITY AND MICROBODIES IN NONHUMAN PRIMATE LIVER[1]

Although urate oxidase (uricase) activity has been biochemically detected in tissues of most vertebrates so far studied, it has not been demonstrated to date in human and nonhuman primates, birds, reptiles and insects (excluding *Diptera*) (Keilin, *Biol. Rev. 34:* 265. 1959; Mahler, *The Enzymes,* Academic Press, Inc., New York, 1963, Vol. 8, p. 285; Baldwin, *An Introduction to Comparative Biochemistry,* Cambridge University Press, Cambridge, 1964, p. 71).

In 1951, Schein, Podber and Novikoff (*J. Biol. Chem. 190:* 331. 1951), noted that, in rat liver homogenates, 75% of urate oxidase activity was present in mitochondrial fractions. In 1955, de Duve *et al.* (*Biochem. J. 60:* 604. 1955) found that urate oxidase activity was in the light mitochondrial layer particularly. Subsequent studies, however, suggested that this enzymatic activity was associated with a different group of particles (de Duve *et al., Biochim. Biophys. Acta 40:* 186. 1960). Baudhuin, Beaufay and de Duve (*J. Cell Biol. 26:* 219. 1965) demonstrated by combined biochemical and electron microscopic studies that microbodies of rats are consistently associated with urate oxidase, catalase and D-amino acid oxidase. Based on morphologic techniques alone, Hruban and Swift (*Science 146:* 1316. 1964) were the first to show the resemblance between the crystalloid of microbodies and uricase crystals. Additional evidence obtained from studies of several species for the correlation between uricase and microbodies displaying a dense core was provided by the comparative studies of Afzelius (*J. Cell Biol. 26:* 835. 1965) and Shnitka (*Fed. Proc. 25:* 361. 1966).

Although it has been stated that hepatic microbodies of man are normally devoid of dense cores, they have been recently found in a liver biopsy from a patient with idiopathic recurrent cholestasis (Biempica and Arias, *Gastroenterology 50:* 395. 1966). In addition, J. U. Balis in our laboratories (personal communication) has observed microbodies with inclusion in human fetal liver (Fig. 1).

The electron microscopic studies of Bearcroft (*J. Path. Bact. 83:* 59. 1962) of livers of two African monkeys (*Cercopithecus aethiops* tantalus and *Erythrocebus patas* patas) are inconclusive regarding the presence or absence of nucleoid in microbodies. On the other hand, no histochemical or electron microscopic information concerning the microbodies of squirrel monkeys (*Saimiri*, Cebidae family) is available at present. Since these platyrrhine monkeys from South America are being increasingly used for laboratory studies, data on their normal morphophysiologic features are needed.

We have recently studied the urate oxidase activity histochemically and the hepatic microbodies in young (sexually immature) male *Saimiri sciureus* monkeys by electron microscopy. Sites of hepatic urate oxidase activity were demonstrated by the technique of Graham and Karnovsky (*J. Histochem. Cytochem. 13:* 448. 1965), with or without urate as substrate at 37°C under air and under 95% oxygen atmosphere for 30–60 min and 15 min respectively. Autopsy and biopsy liver specimens were processed for electron microscopy.

In sections incubated with urate under air, the

[1] Supported by a grant from the Medical Research Council of Canada (Fund MA1904).

reaction was positive at 30–60 min. The reaction product was evidenced by brown granules of small size sparsely distributed in the cytoplasm of hepatocytes. No preferential distribution was observed either around the nucleus within cells or in any particular zone within liver lobules, (Fig. 2). After 15 min incubation under 95% oxygen atmosphere with urate in the medium, the reaction product was similar to that obtained by 30–60 min incubation of slices in air. Control sections incubated in media without urate showed no reaction under similar conditions (Fig. 3).

Ultrastructural studies of the hepatocytes showed microbodies (0.4–1.0 μ) randomly distributed within the cytoplasm in close relation with the endoplasmic reticulum (Fig. 4). The most conspicuous features of microbodies were as follows. (1) The matrix was moderately dense and presented a nucleoid of higher electron density. (2) The shape and size of these inclusions were irregular, although in most of the sections they were seen as bars traversing the matrix in straight or curving forms close to the limiting membrane or in other instances occupying the center of the particles (Figs. 5–7). This type of nucleoid was similar to those observed in cows and in human fetal or pathologic liver (*loc. cit.*). (3) Inclusions had a periodicity of about 110 A (Fig. 8).

Under the conditions of these observations, we conclude that urate oxidase activity is present in the liver of the normal squirrel monkey. It is most probably associated with microbodies which do have a dense core. Although there are no available data on the structure of hepatic microbodies of other species of monkeys for comparative purposes, we suggest the exclusion of the *Saimiri sciureus* from the classical division of mammals which do not contain uricase. It is intriguing to observe that the Dalmatian coachhound, whose purine metabolism presents a peculiar behavior (Wolfson, Cohn and Shore, *J. Exp. Med. 92:* 121. 1950), has microbodies which resemble some of the morphologic features found in squirrel monkeys (Afzelius, *loc. cit.*).

April 28, 1966

FELIX A. DE LA IGLESIA[2]
EDUARDO A. PORTA
W. STANLEY HARTROFT
The Research Institute of the Hospital for Sick Children
Toronto, Canada

[2] Research Fellow, Medical Research Council of Canada. Present address: Warner-Lambert Research Institute, Sheridan Park, Clarkson, Ontario, Canada.

FIG. 1. Microbody with dense core (arrow) in a 6-week gestation human fetal liver. The nucleoid is off center and displays a typical periodicity. (Photograph by courtesy of Drs. J. U. Balis and P. E. Conen.) Lead stain, ×49,500.

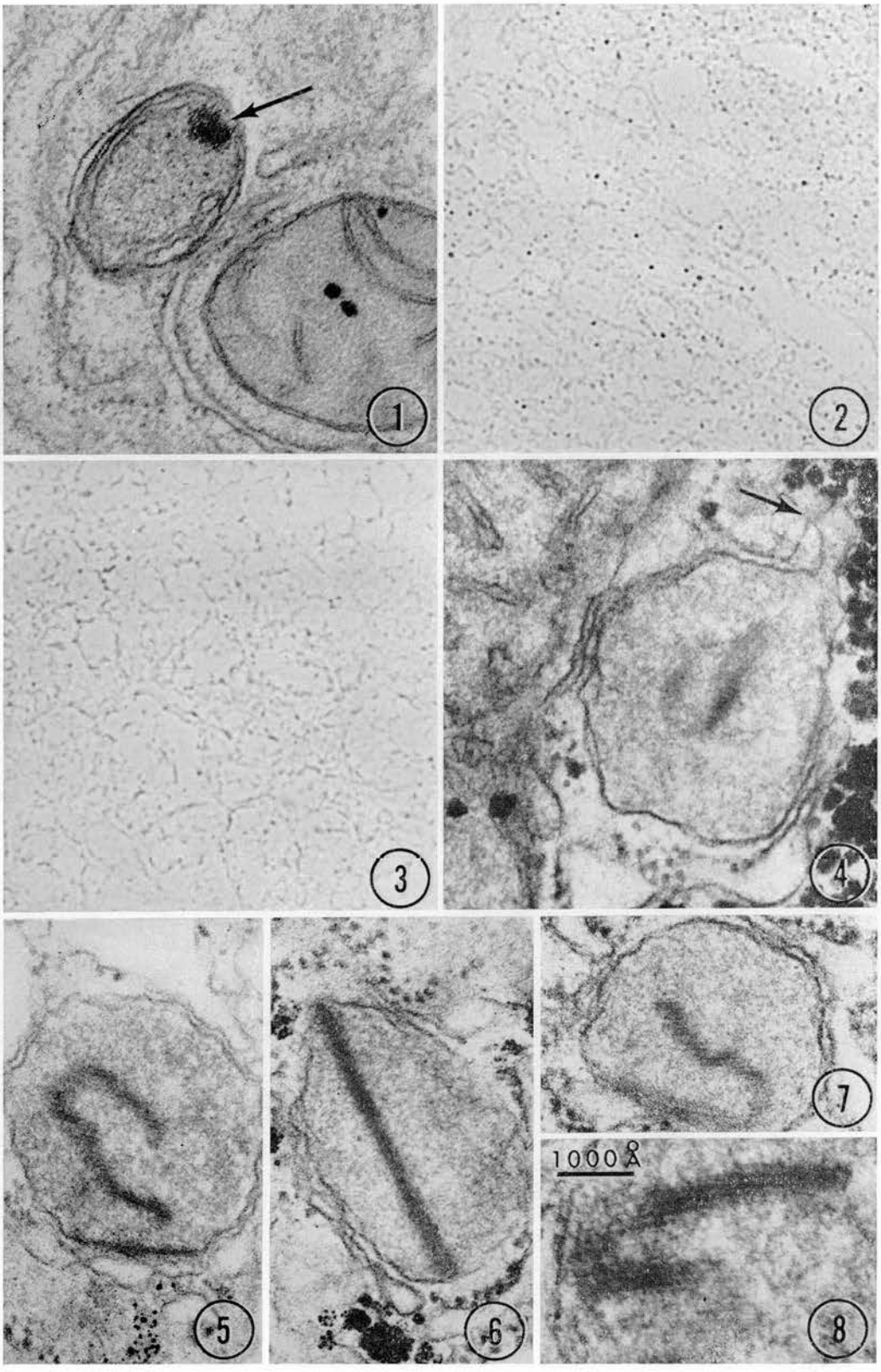
FIG. 2. Urate oxidase reaction product (dark granules); section incubated with urate for 60 min under air atmosphere. There is no particular arrangement of the particles in the cytoplasm. Unstained section, ×500.

FIG. 3. Adjacent section to that shown in Figure 2; the tissue was treated as above, but urate was omitted from the incubation medium. No reaction product is seen. Unstained section, ×500.

FIG. 4. Microbody of squirrel monkey displaying a central core. The tail-like prolongation shows the connection with the endoplasmic reticulum (arrow). Lead stain, ×73,200.

FIGS. 5–7. These electron micrographs illustrate some of the variations found in microbodies of squirrel monkeys. All nucleoids exhibit periodicity. Lead stain, ×55,200, ×73,200 and ×73,200 respectively.

FIG. 8. Electron micrograph showing part of a nucleoid at high magnification, in which a spacing of about 110 A can be appreciated. Lead stain, ×122,000.



FIGS. 1–8

AUTORADIOGRAPHIC CHARACTERIZATION OF SULFATED ACID MUCOPOLYSACCHARIDES IN EXPERIMENTAL CIRRHOSIS[1]

The demonstration that various sulfated mucopolysaccharides can be localized in tissue sections by combining alcian blue staining with various enzyme digestions (Zugibe, *J. Histochem. Cytochem. 10:* 441. 1962) suggested that this might be a valuable tool to analyze changes in the composition of the hepatic ground substance during the induction of experimental cirrhosis. However, alcian blue staining in the liver was so faint, even in well developed fibrous septa, that we were unable to interpret it.

Since $^{35}SO_4$ is taken up in fibrous septa in carbon tetrachloride-induced cirrhosis in the rat (Patrick, and Kennedy, *J. Path. Bact. 88:* 549. 1964), we attempted to characterize septal mucopolysaccharides by means of enzyme treatment and autoradiography. Cirrhosis was produced in 3 female Sprague-Dawley rats of 150 g initial weight by biweekly injections of 0.1 ml CCl_4 in olive oil per 100 g body weight for 9 weeks. One mc of $Na_2{}^{35}SO_4$, specific activity 195 mc/*mM* (New England Nuclear Corp.), was injected intraperitoneally, and the animals were killed 72 hr later. The livers were perfused with cold saline. Small blocks of liver and trachea were frozen in isopentane at −70°C. Sections were cut at 5 μ in a Harris cryostat and fixed for 2 hr in 10% neutral buffered formalin containing 1% cetylpyridinium chloride. The sections were washed in water and incubated for 24 hr at 37°C in two changes of a solution of 100 U.S.P. units of testicular hyaluronidase (Sigma Chemical Company) per ml of 0.1 *M* acetate buffer, pH 5.0, with 0.1 *M* NaCl added. Control sections were incubated with buffer alone. The sections were then washed in distilled water and dipped in 0.5% gelatin. Autoradiograms, using Kodak NTB-3 nuclear track emulsion, were prepared by standard techniques, with an exposure of 80 days.

The sections of trachea showed very heavy labeling over the cartilaginous rings (Fig. 1A). Treatment with testicular hyaluronidase removed almost all radioactivity (Fig. 1B). In the liver the septa were conspicuously labeled (Fig. 2A). After treatment with hyaluronidase about one-third of the label over the septa remained (Fig. 2B). Chondroitin sulfates A and C are digested by testicular hyaluronidase, while chondroitin sulfate B, heparitin sulfate and keratosulfate are resistant. Since hyaluronidase treatment removed the label from tracheal cartilage, the newly synthesized mucopolysaccharides in this tissue are chondroitin sulfate A and/or C. On the other hand, cirrhotic septa contain not only chondroitin sulfates A and/or C, but also a considerable moiety of newly synthesized chondroitin sulfate B, heparitin sulfate, keratosulfate or a combination of these. Although heparin sulfate is also resistant to hyaluronidase, it seems unlikely that it localizes in cirrhotic septa, except in mast cells. Further experiments using bacterial enzymes specific for individual sulfated mucopolysaccharides are necessary, however, to categorize the septal ground substance more exactly.

In the study of experimental cirrhosis, the advantages of autoradiography combined with enzyme digestion over staining techniques are its much greater sensitivity and perhaps easier quantitation. It differs in demonstrating only mucopolysaccharides synthesized at the time $^{35}SO_4$ is given, rather than the total amount present.

[1] Supported by Research Grant AM-03846 from the National Institutes of Arthritis and Metabolic Diseases, U.S. Public Health Service.

May 5, 1966

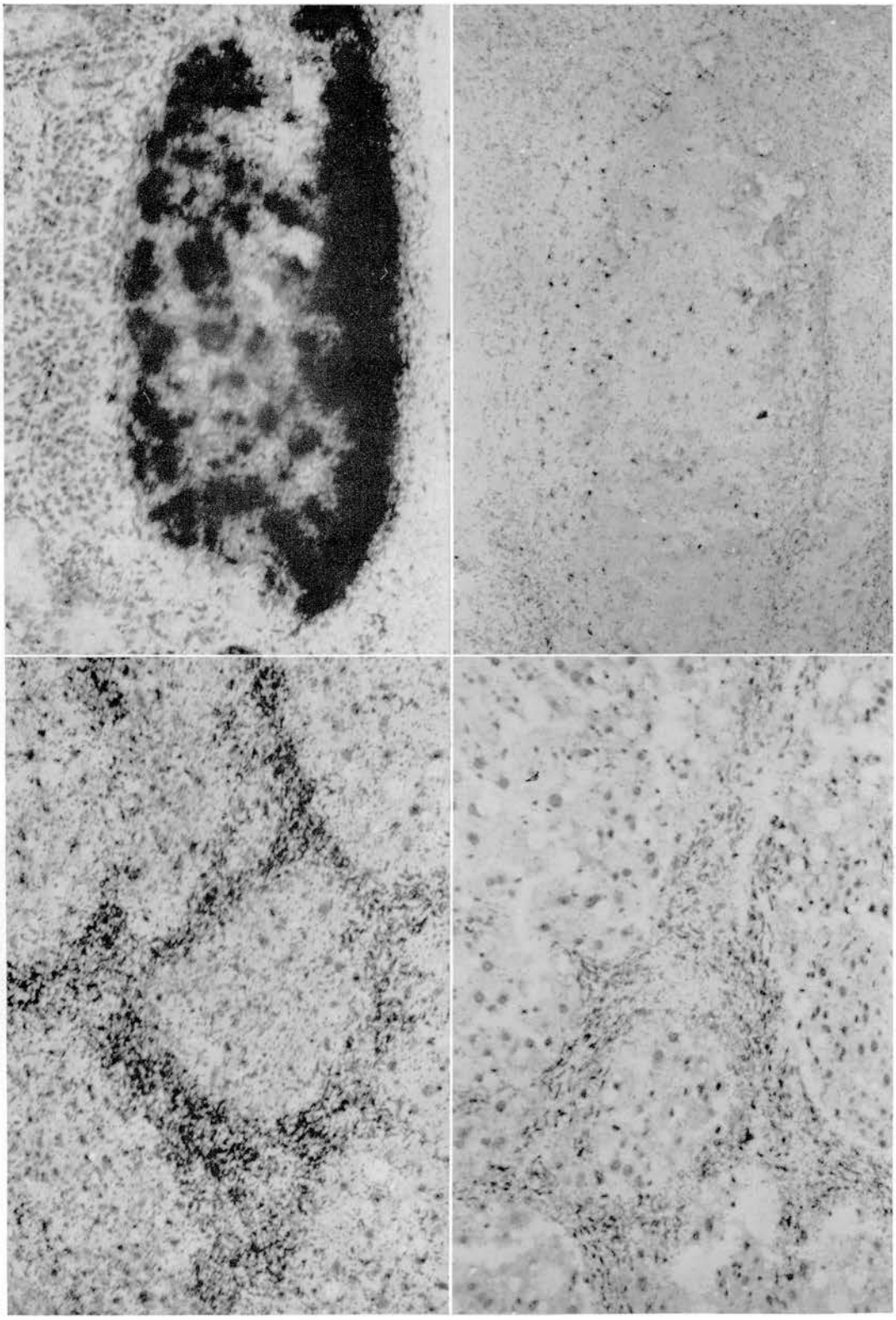
EMANUEL RUBIN
Department of Pathology
Mount Sinai School of Medicine
New York, N.Y.

FIG. 1A (upper left). Autoradiogram of tracheal cartilage from a rat killed 72 hr after the injection of $^{35}SO_4$ showing heavy labeling. ×240.

FIG. 1B (upper right). Autoradiogram of tracheal cartilage from a rat killed 72 hr after the injection of $^{35}SO_4$ showing loss of radioactivity after treatment of section for 24 hr with testicular hyaluronidase. ×240.

FIG. 2A (lower left). Autoradiogram of cirrhotic liver from a rat killed 72 hr after the injection of $^{35}SO_4$ showing labeling over septa. ×240.

FIG. 2B (lower right). Autoradiogram of cirrhotic liver from a rat killed 72 hr after the injection of $^{35}SO_4$ after treatment of sections for 24 hr with testicular hyaluronidase. The intensity of label is reduced, but a significant amount of radioactivity remains. ×240.



FIGS. 1–2

DEMONSTRATION OF CATECHOLAMINES IN ADRENERGIC NERVE FIBERS BY FIXATION IN AQUEOUS FORMALDEHYDE SOLUTION AND FLUORESCENCE MICROSCOPY[1]

Fixation in an aqueous solution of formaldehyde and subsequent fluorescence microscopy is a convenient method for the histochemical demonstration of noradrenaline in the adrenal medulla (Eränkö, *Acta Endocrinol. 18:* 174. 1955). However, this method fails to demonstrate noradrenaline in adrenergic nerve fibers, although these become intensely fluorescent when exposed dry to formaldehyde vapor (Falck, *Acta Physiol Scand. 56:* Suppl., 198. 1962). Using this modification, we observed (Eränkö and Räisänen, in *Mechanisms of Release of Biogenic Amines*, edited by Euler, Uvnäs and Rosell, Oxford, Pergamon Press, 1966) that the Krebs-Ringer Solution I (Long, *Biochemists' Handbook*, London, E. & F. N. Spon, Ltd., 1961, p. 58), adjusted at pH 7.0 and supplemented with 0.1 mg/ml calcium chloride and 1 mg/ml glucose, is a medium in which iris can be incubated for hours without loss or dislocation of nervous noradrenaline. We have now used this solution as a solvent for formaldehyde, with good results.

Fresh preparations of rat iris were immersed in 0.1–3.2% formaldehyde solution made in the above mixture. After fixation for 30 min–18 hr at 4°C, the iris was stretched on a clean slide and it was allowed to dry. It was then examined in the fluorescence microscope.

Figure 1 shows a preparation fixed for 30 min in a 0.35% formaldehyde solution. The adrenergic nerve net with its intensely fluorescent varicosities is visible exactly in the same way as after treatment with formaldehyde vapor. The formaldehyde concentration was not critical. However, 1.6 and 3.2% formaldehyde produced a weak fluorescence. Fixation for 2 hr resulted in a weak fluorescence with all concentrations, and no fluorescence was detected after fixation overnight.

Some experiments were carried out by perfusion with ice-cold formaldehyde solutions. The nerve net of the iris became fluorescent after intra-arterial perfusion with 1% formaldehyde, but

[1] Supported by grants from the State Medical Commission, from the Sigrid Juselius Foundation (to O. E.) and from the Yrjö Jahnsson Foundation (to L. R.).

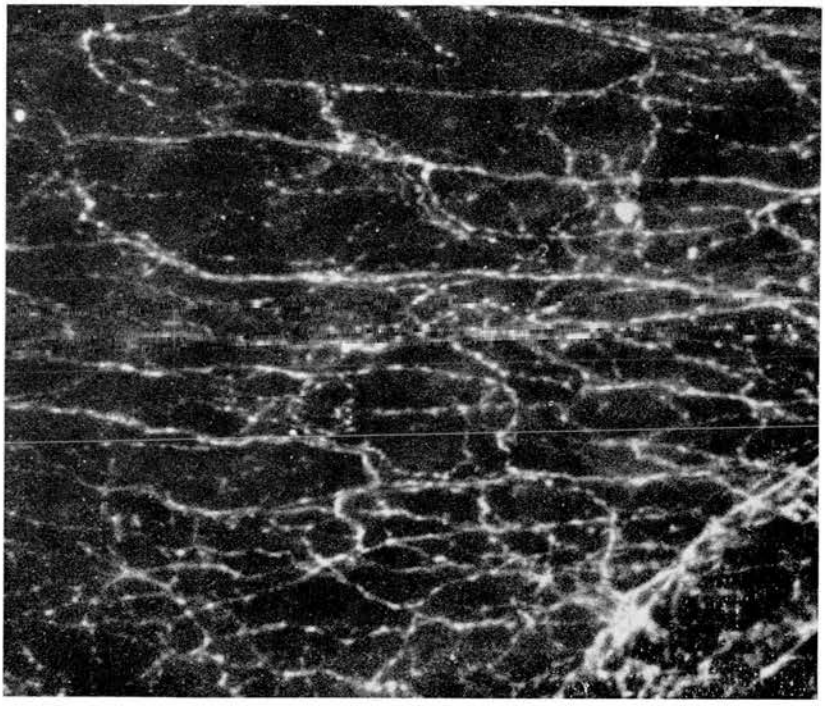


FIG. 1. Whole mount of rat iris fixed for 30 min in 0.35% formaldehyde solution made in Krebs-Ringer fluid at 4°C. Fluorescence photomicrograph. Noradrenaline-containing nerve fibers innervating the dilator muscle are brightly fluorescent. An arteriole in the lower right corner. ×240.

failed to do so if the concentration was 0.35%. However, the catecholamines were not displaced by this treatment, as was shown by treating the iris with formaldehyde vapor.

The observations presented show that a short fixation in an aqueous solution of formaldehyde is capable of converting intraneuronal catecholamines into intensely fluorescent compounds without causing their dislocation. This is of considerable interest from the point of view of general fixation mechanisms and concerning the fluorescence reaction of catecholamines. Furthermore, the method is useful in practice by causing much less inactivation of enzymes than treatment with formaldehyde vapor. This is valuable in the study of enzymes such as acetylcholinesterase which are sensitive toward formaldehyde vapor.

July 14, 1966

Olavi Eränkö
Liisa Räisänen
Department of Anatomy
University of Helsinki
Siltavuorenpenger
Helsinki, Finland

INDOLE REACTIONS OF ENTEROCHROMAFFIN CELLS AND MAST CELLS

At present, the identification of the enterochromaffin substance as 5-hydroxytryptamine (Erspamer and Asero, *Nature 169:* 800. 1952) contrasts with the fact that, by applying several histochemical methods to formalin-fixed sections, no indole reactions of enterochromaffin cells have been observed (Lillie, *J. Histochem. Cytochem. 9:* 184. 1961). We have reconsidered the problem by fixing thin specimens of duodenum, stomach and pancreas of various species, including guinea pig, rat, mouse, rabbit, horse, pig, monkey and man, with 4% formaldehyde or with 6% glutaraldehyde in 0.1 *M* Sörensen's phosphate buffer, pH 6.5–7.4 for 24 hr at room temperature.

In all species examined, both formaldehyde- and glutaraldehyde-fixed enterochromaffin cells of duodenum and stomach gave positive diazonium (fast garnet GBC 1 mg/ml in 0.1 *M* phosphate buffer at pH 7.8, for 2 min), Masson-Hamperl and ferric-ferricyanide reactions. Hence, enterochromaffin substance appeared to be well preserved by both aldehydes, although stronger reactions were obtained with glutaraldehyde than with formaldehyde. By applying to formaldehyde-fixed tissues the dimethylaminobenzaldehyde method for indoles according to Lison's technic (*Histochimie et Cytochimie Animales*, Gauthier-Villars, 1960) with or without nitrite post-treatment, the "post-coupled benzylidene" method according to Glenner and Lillie (*J. Histochem. Cytochem. 5:* 279. 1957) and the xanthydrol method according to Lillie (*J. Histochem. Cytochem. 5:* 188. 1957), enterochromaffin cells were never stained, whereas zymogen granules in the pancreas and stomach as well as Paneth cells granules and other tryptophan-containing structures were well stained. Conversely, in the glutaraldehyde-fixed tissues enterochromaffin granules were clearly reactive to the indole tests (Fig. 1); namely, these granules stained deep blue-green by

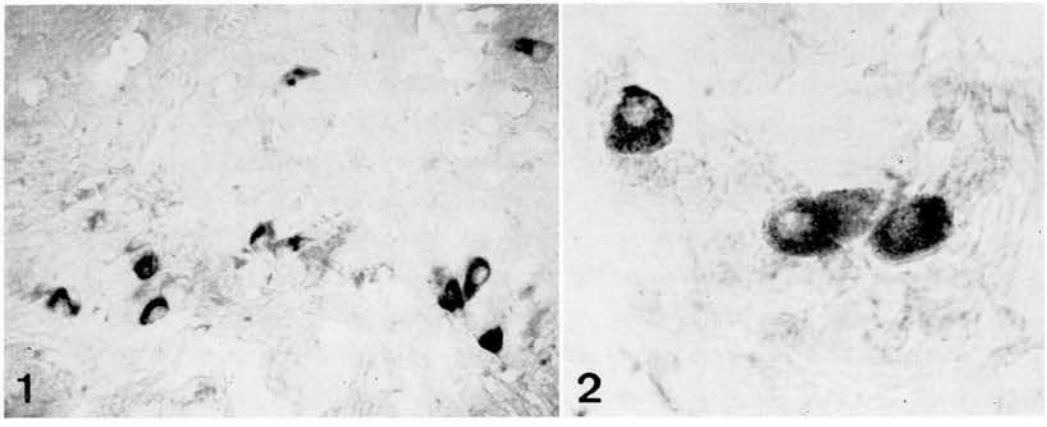


Fig. 1. Guinea pig enterochromaffin cells in glutaraldehyde-fixed duodenal mucosa, stained with xanthydrol. ×320.
Fig. 2. Rat mast cells in glutaraldehyde-fixed gastric submucosa, stained with xanthydrol. ×1200.

xanthydrol, blue by dimethylaminobenzaldehyde and dark blue-yellow both by dimethylaminobenzaldehyde-nitrite and the post-coupled benzylidene reactions. In the same sections tryptophan - containing structures were stained purple-violet with xanthydrol or dimethylaminobenzaldehyde and blue with dimethylaminobenzaldehyde - nitrite or the post-coupled benzylidene reactions. These results suggest that glutaraldehyde reacts differently than formaldehyde with tissue indoles. Formaldehyde fixes both 5-hydroxytryptamine and protein-bound tryptophan, but completely blocks the reactivity of the 2 (α)carbon in the 5-hydroxytryptamine pyrrole ring, while it does not greatly affect the same group in protein-bound tryptophan, at least after a 24-hr treatment. On the other hand, glutaraldehyde seems not to modify the histochemical reactivity of either tryptophan or 5-hydroxytryptamine.

By employing the xanthydrol method it was easy to distinguish greenish blue stained enterochromaffin granules from purple-violet zymogen granules. *In vitro* tests have been performed in order to clarify the significance of this histochemical finding. When 1 mg isatin, histamine, adrenaline, noradrenaline, dihydroxyphenylalanine (dopa) or dihydroxyphenylethylamine (dopamine) was added to 1 ml 0.5% xanthydrol solution in 9:1 acetic acid-hydrochloric acid mixture, a heavily colored reaction was not observed; conversely, with 5-hydroxytryptamine a dark blue-green color with 5-hydroxytroptophan a dark green-blue, with tryptamine and tryptophan a purple-violet color was obtained. In dilute solutions the color developed by 5-hydroxytryptamine or tryptophan was similar to the one observed in enterochromaffin or zymogen granules respectively in tissue sections.

Spectrophotometrically the reaction products of 5-hydroxyindoles with xanthydrol showed an absorption maximum at 580–590 mμ, whereas those of unsubstituted indoles showed a maximum at 510–520 mμ; by histospectrophotometry of tissue sections stained with Lillie's xanthydrol method, comparable absorption maxima have been obtained in enterochromaffin and zymogen granules, respectively. In another experiment 10-mg samples of 5-hydroxytryptamine, 5-hydroxytryptophan, tryptamine, tryptophan, adrenaline, noradrenaline, histamine, dihydroxyphenylalanine (dopa) and dihydroxyphenylethylamine (dopamine) were incubated for 24 hr with 5 ml 6% glutaraldehyde buffered at pH 7.2. Various amounts of precipitates were obtained with 5-hydroxytryptamine, tryptamine, noradrenaline, dopamine, 5-hydroxytryptophan, tryptophan and dopa, but not with adrenaline and histamine. All precipitates, when tested with xanthydrol, reacted similarly to the parent substance. Thus, the xanthydrol method seems to offer a simple and useful histochemical test for 5-hydroxytryptamine, tryptophan and some related compounds. In our opinion, the above results give direct histochemical evidence of the indole nature of the enterochromaffin substance and strongly support Erspamer's 5-hydroxytryptamine theory.

It is worth noting that formaldehyde-fixed mast cells in tissue sections of all species examined behaved like tryptophan-containing structures or appeared unstained. In glutaraldehyde-fixed sections the mast cells of man, monkey, guinea pig, rabbit, hamster and pig stained as after formaldehyde-fixation, while mast cells of rat and mouse reacted similarly to enterochromaffin cells (Fig. 2). Actually, in these species the mast cells were stained a deep blue by xanthydrol and blue by dimethylaminobenzaldehyde while in other species they were stained a light purple-violet with these methods and were somewhat more intensively stained by dimethylaminobenzaldehyde-nitrite or the post-coupled benzylidene reaction, just as protein-bound tryptophan. Also, the rat and mouse mast cells appeared to be clearly reactive to the diazonium, Masson-Hamperl and ferric-ferricyanide methods, which were considerably improved by glutaraldehyde fixation; conversely, in mast cells of other species there was no significant reaction to these methods either in formaldehyde- or in glutaraldehyde-fixed tissues. These findings support the hypothesis that 5-hydroxytryptamine is stored in rat and mouse mast cells (Benditt *et al.*, *Proc. Soc. Exp. Biol. Med. 90:* 303, 1955; Sjoerdsma *et al.*, *Science 125:* 1202, 1957). It should be noted, however, that great differences were observed from one cell to another in the reactivity to histochemical tests, suggesting marked differences in the amount of 5-hydroxytryptamine stored in the cytoplasmic granules.

June 16, 1966

Enrico Solcia
Roberto Sampietro
Gabriele Vassallo
Department of Pathological Anatomy
The University of Pavia
Pavia, Italy

Reprinted from
ANNUAL REVIEW OF PHARMACOLOGY
Vol. 7, 1967

HISTOCHEMISTRY OF NERVOUS TISSUES: CATECHOLAMINES AND CHOLINESTERASES[1,2,3]

By Olavi Eränkö

Department of Anatomy, University of Helsinki, Helsinki, Finland

Histochemical methods have made it possible to explore the chemical mechanisms of nervous transmissions at the cellular level. The introduction by Koelle (130) of a reliable method for histochemical demonstration of cholinesterases greatly facilitated the study of the cholinergic mechanisms and led to a new concept of cholinergic transmission (133). Histochemical methods for the demonstration of catecholamines were improved only a few years ago to a degree permitting the study of these amines in the nervous tissue.

A large number of publications has accumulated on both subjects. Within the space allotted to this review it is impossible to refer to all of them, but the studies cited provide further references, and these virtually cover the whole field. A special effort has been made to give fair balance to the recent observations, which are truly stimulating and give promise of a rapid further development.

Catecholamines

Light microscopic demonstration.—Eränkö (55, 57) observed about ten years ago that following the exposure of sections of the adrenal medulla to formaldehyde solution, a fluorescence developed in some cells, which were shown with specific chemical methods to be norepinephrine-containing. Since it was also found that norepinephrine forms a fluorescent compound with formaldehyde, treatment with this compound was proposed to be a histochemical method for the demonstration of norepinephrine. Although the fluorescence was stronger in sections dried after exposure to formaldehyde (58), the method was not sufficiently sensitive for localizing catecholamines in adrenergic nerve fibers. However, it was later observed that a very intense fluorescence appeared when monoamines in freeze-dried tissues were exposed to formaldehyde vapor (64, 86, 140). A successful modification based on this principle was described in the publications of Falck, Hillarp and associates (80, 82). In this connection it is of interest to note that the adrenergic nerve net of the iris can be equally well demonstrated by treatment in a solution of formaldehyde, made in Ringer's fluid so as to avoid osmotic damage, as by exposure to formaldehyde vapor (74).

[1] The survey of the literature pertaining to this review was concluded in June 1966.

[2] The following abbreviations will be used: 5-HT (5-hydroxytryptamine); ACh (acetylcholine).

[3] Work supported by a grant from the Sigrid Juselius Foundation, Helsinki.

Reaction mechanism and specificity.—Corrodi and Hillarp observed that DOPAmine and formaldehyde condense with the formation of 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, which is then dehydrogenated into an intensely fluorescent compound, 6,7-dihydroxy-3,4-dihydroisoquinoline (27, 28). Dihydroxyphenylalanine, norepinephrine, and epinephrine are similarly converted into fluorescent compounds. 5-Hydroxytryptamine (5-HT) condenses into 6-hydroxy-3,4-dihydro-β-carboline, which is also intensely fluorescent (31).

Disappearance of the formaldehyde-induced fluorescence after treatment with sodium borohydride (29), which reduces the dihydroisoquinolines back into nonfluorescent tetrahydroisoquinolines, is highly suggestive of monoamines, especially if the fluorescence can be regenerated by repeated exposure to formaldehyde. However, specific chemical analysis is always valuable, and should be carried out if there is any doubt of the nature of the fluorogenic substance.

Discrimination between amines.—Isoquinoline derivatives from catecholamines generate a green fluorescence with an emission maximum at 4800 Å, while the fluorescent compound obtained from 5-HT is yellow with an emission maximum at 5300 Å (31). These spectral differences make it possible to discriminate between catecholamines and 5-HT.

Epinephrine, being a secondary amine, is more slowly condensed with formaldehyde than DOPAmine or norepinephrine and can thus be discriminated from these (61, 80). Corrodi & Jonsson (30) found that the hydroxy-group at the position 4 in the 3,4-dihydroisoquinoline derivative from norepinephrine can be split off by treatment with thionyl chloride with formation of the fully aromatic 6,7-dihydroxyisoquinoline. This compound retains its fluorescence after treatment with sodium borohydride, in contrast to 6,7-dihydroxy-3,4-dihydroisoquinoline formed by formaldehyde condensation from DOPAmine, which is reduced by thionyl chloride into nonfluorescent 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (30).

Practical procedure.—In practice, the method is simple to perform but all the steps are somewhat critical. Methodological aspects have recently been dealt with in several papers (40, 61, 80, 82, 84, 107, 125). Four steps are involved: (*a*) freeze-drying, (*b*) exposure to formaldehyde, (*c*) embedding, sectioning, and mounting, and (*d*) fluorescence microscopy.

Freezing must be rapid to avoid ice-crystal artifacts (56, 164). It is usually carried out by immersion in isopentane cooled with liquid nitrogen (164), but metal surfaces precooled in liquid nitrogen provide more rapid cooling (56, 116). Drying must occur at a temperature below −35° C, otherwise diffusion of the amine to be demonstrated destroys the localization [for methods see (164, 188)].

The tissues have usually been embedded in paraffin wax but sections embedded in an epoxy resin can be cut thinner, which improves the localizing power of the method (119).

Formaldehyde vapor is generated from paraformaldehyde powder, whose

water content must first be standardized by storage in a desiccator over sulfuric acid to create an atmosphere of suitable relative humidity (114). Besides the relative humidity, the temperature and the length of exposure also influence the reaction; a rule of thumb is that the sum of humidity and temperature (e.g., 70 per cent plus 40° C) must be approximately 110 (63).

A good microscope lamp with a high-pressure mercury burner such as HBO 200 is essential for fluorescence microscopy but, against a widespread misconception, quartz optics are not necessary or even desirable.

Amine concentration and fluorescence intensity.—While the intensity of the fluorescence induced by treatment with formaldehyde solution bears a linear relationship with the catecholamine concentration (71), recent observations indicate that the same does not apply to the intensity of the fluorescence induced by formaldehyde vapor, the intensity remaining the same or even decreasing above certain amine concentrations (172).

Neuronal distribution.—Distribution of the amines has been extensively studied with this method in the different parts of the aminergic neurons. As could be expected from earlier chemical observations by Von Euler [see (77)], the nerve cell bodies have been found to exhibit a moderate fluorescence, a weak fluorescence is seen in the preterminal axons, while the terminal nerve fibers and, especially, the synapses exhibit an intense fluorescence [e.g. (40, 149, 158)].

Sympathetic ganglia.—A diffuse fluorescence of variable intensity was observed by Eränkö & Härkönen (65) in the cytoplasm of all ganglion cells of the superior cervical ganglion of the rat. However, brilliantly fluorescent small granules were observed in the cytoplasm of many cells and in the fibers between the cells. The observations were understood to suggest that, in addition to a diffuse soluble pool, there is a pool of catecholamines concentrated in granules, which migrate from the perikaryon to the axon. This view has been strongly opposed by Norberg & Hamberger (158) who maintain that the cytoplasmic amine fluorescence is always diffuse; the granules, if any, cannot be resolved by light microscopy. The matter should be further examined, possibly by the conjoint use of fluorescence and electron microscopes.

Norberg & Hamberger (108, 112, 157) made the highly interesting observation that there are large numbers of intensely fluorescent synapses in many sympathetic ganglia, apparently in direct axosomatic contact with fluorescent ganglion cell bodies. Because neither preganglionic nor postganglionic nerve division had any effect on these terminals, they were concluded to originate from cell bodies present in the same ganglion, either from intraganglionic interneurons or from collaterals of the postganglionic fibers (108). The former alternative is especially attractive in view of the previously observed adrenergic inhibition of synaptic transmission in the sympathetic ganglia (32).

Small cells which exhibit a much more intense fluorescence than ganglion cells were first reported by Eränkö & Härkönen (65, 68) in the superior cervical ganglion of the rat. Since the cells were nonchromaffin, looked electron

microscopically like adrenal medullary cells, and their fluorescence appeared yellow, it was tentatively concluded that they perhaps contained 5-HT. It now seems more likely that DOPAmine is responsible, because the fluorescence spectrum of these cells is typical of a catecholamine (173). Similar small, intensely fluorescent cells have also been reported in other ganglia and nerve bundles (158, 162, 183).

Peripheral innervation apparatus.—As described first in Falck's (80) paper, the peripheral end of the sympathetic neuron has been observed to consist of a fine, fluorescent nerve fiber which ramifies and forms, with branches of other fibers, a network of terminal axons. Along the 0.2 μ thick terminal axons there are at regular intervals varicosities, 0.5–1 μ thick and 1–2 μ long, which exhibit an intense formaldehyde-induced fluorescence. Several varicosities are seen along each fiber, and they seem to be synapses of the "boutons de passage" type. The amine histochemistry of the peripheral neurons has recently been reviewed by Falck & Owman (83). The iris has been a favorite organ for studying the peripheral nerve net, because the amine fluorescence of its nerve fibers can be easily studied in fresh stretch preparations. Adrenergic terminals have been repeatedly found not only in the dilator (87) but also in the sphincter muscle (149).

Ramification of peripheral axons.—Crushing the superior cervical ganglion with tweezers, so as to leave but a few ganglion cells intact, has been ingeniously performed by Malmfors & Sachs (150) to study the mode of ramification of the adrenergic fibers in the peripheral innervation apparatus of the iris. It was reported that a single adrenergic preterminal axon may ramify and send richly branched terminal fibers into (*a*) the ciliary body, (*b*) an arteriole, (*c*) the dilator, and (*d*) the sphincter muscles of the iris. This is a most interesting observation, but it cannot be quite excluded that what appears in the light microscope as a single axon, may in fact be a group of axons running closely together but each separately innervating one of the four tissues. Electron microscopic examination of the preterminal fiber after fluorescence microscopy could settle this problem.

Innervation of different organs.—An extensive study on the iris has been published by Malmfors (149), and other ocular and orbital structures have been reviewed by Ehinger (51). The presence of DOPAminergic neurons and fibers in the inner plexiform layer of the retina (52, 104) is of special interest.

Both the atrial and the ventricular muscles of the heart and, especially, its atrioventricular node are supplied with adrenergic networks (7, 8, 81). On the other hand, the adrenergic fibers of the skeletal muscle are limited to blood vessels (95). Serous acini of the submandibular and parotid glands are richly innervated by adrenergic fibers, while the mucous acini of the sublingual gland contain none (4, 159).

The pineal body has been thoroughly studied by Owman and co-workers (14, 105, 160, 161). The pineal parenchymal cells contain histochemically demonstrable 5-HT, and they are innervated by noradrenergic fibers originating from the superior cervical ganglion. Curiously, these fibers contain not

only norepinephrine but also 5-HT, apparently taken up from the parenchymal cells.

A rich adrenergic innervation has been found in almost all organs which contain smooth muscle: the iris (see above); the arteries, the arterioles, and the veins (95), which are especially densely innervated in the erectile tissues of the nasal mucosa (43) and the penis (70, 166); hair arrector muscles (85, 93); the nictitating membrane (125); the bronchial muscle (45); the trabecular network of the spleen (47, 99); the vas deferens, the seminal vesicle, and the prostate (125, 162, 183); as well as the oviduct and the uterus (17–19, 125).

However, the smooth muscle is not everywhere supplied with monoamine-containing fibers. Norberg (156) observed that the adrenergic postganglionic fibers of the intestine form terminal networks around the cholinergic cell bodies of the intramural ganglia only; the smooth muscle is innervated exclusively by cholinergic terminals originating from these cells.

Hamberger & Norberg (109) found intramural ganglia also in the bladder, in which some of the ganglion cell bodies were catecholamine-containing and the others nonfluorescent but surrounded by an adrenergic nerve net. Only in the trigone area was the smooth muscle supplied with adrenergic nerve fibers; elsewhere in the bladder muscle adrenergic nerves were found in the blood vessels only.

The innervation of the vas deferens, the seminal vesicle, and the prostate likewise originates from a multitude of small peripheral ganglia scattered around the internal genitals, as was observed by Owman & Sjöstrand (162) and by Sjöstrand (183). These ganglia supply the genitals with a dense adrenergic nerve net and their catecholamine content is therefore unaffected by hypogastric denervation. In the vas deferens, the seminal vesicles, and the prostate, the same authors (162, 183) found clusters of small, intensely fluorescent cells, apparently chromaffin cells which contained epinephrine. Chromaffin cells were earlier described in the hypogastric nerves by Vanov & Vogt (191), and their presence explains many apparently anomalous results obtained in depletion experiments.

Central aminergic neurons.—The pioneer work by Vogt (192) and Amin, Crawford & Gaddum (1) showed that monoamines are present in the central nervous system; it was then of obvious interest to investigate their microscopic distribution in the brain. Fine varicose fibers showing an intense fluorescence were indeed found already in the first histochemical studies of the central nervous system by Carlsson et al. (25, 26), while fluorescent nerve cell bodies were not observed. Subsequent extensive studies, notably by Andén, Dahlström, and Fuxe, who have published detailed data, have indicated that the cell bodies of the monoamine-containing neurons are almost exclusively localized in the brain stem, from which originate the ascending aminergic pathways to the higher brain nuclei and the descending pathways to the spinal cord (2, 3, 40, 42, 90, 91). For details, the original papers should be consulted.

Several large neuron systems have been described using the fluorescence method. Thus, norepinephrine- and 5-HT-containing terminals have been found in the spinal cord and traced to definite cell groups in the medulla oblongata (42). The 5-HT-containing terminals surrounding the preganglionic sympathetic cells in the lateral horn of the spinal cord, which innervate the adrenal medulla, have been proposed to have an inhibitory function (2). Further, two large ascending neuron systems have been reported, one of which is composed of DOPAmine-containing neurons originating in the substantia nigra and supplying the neostriatum with a vast number of terminals (3). The other, which is mainly noradrenergic, forms extensive ipsilateral mesencephalo-hypothalamic, mesencephalo-limbic, and mesencephalocortical pathways (40).

Median eminence is the only area above the mesencephalon whose fluorescent terminals are not affected by ipsilateral transection of the crus cerebri and the medial forebrain bundle; its fibers have been traced to tuberoinfundibular cell bodies (90).

In the area postrema, long known for its particularly high content of norepinephrine and 5-HT (1, 192), both green and yellow fluorescent nerve cell bodies and green fibers were found, thus providing the explanation for the presence of both amines (94).

Neurons of nonvertebrates.—Monoamine-containing neurons have been described not only in vertebrates but also in other animals, including quite primitive ones. Thus, numerous catecholamine-containing neurons were found in the cockroach brain by Frontali & Norberg (89). Adrenergic sensory cells in mollusks, turbellaria, earthworms, and leeches were observed by Dahl et al. (37), and adrenergic neurons with a combined sensory and motor function were reported in sea anemones by the same authors (38).

Nerve division and axon reaction.—Several studies have shown that division or compression of a peripheral or central adrenergic axon results in rapid accumulation of fluorogenic amines in the proximal side of the lesion and disappearance of the fluorescence from the peripheral part of the neuron, including the nerve terminals (16, 39, 41, 42, 67, 115, 128). The accumulation of amines in the proximal part of the neuron has indeed made it possible to make intensely fluorescent such central aminergic fibers both in the central (42) and the peripheral (41) nervous system, which are not normally demonstrable because of the low amine concentration in the nonterminal axon. It is of interest that some fluorescent material may accumulate also at the peripheral side of the lesion, which suggests the presence of ascending adrenergic fibers in peripheral nerves (39).

While accumulation of amines in the proximal stump has always been observed after transection of the axon, such operation has different effects on the fluorescence of the cell body from which it originates, depending on the site of the division. Härkönen (115) reported that division of the postganglionic nerves near the ganglion causes a complete loss of amine fluorescence from all the ganglion cells of the superior cervical ganglion. On the

other hand, Owman (160) found after pinealectomy an increase in the fluorescence intensity of some cells in the same ganglion. Dahlström & Fuxe (42) reported that the transection of aminergic pathways in the spinal cord likewise resulted in a clear retrograde increase of the fluorescence in the corresponding nerve cell bodies; this phenomenon can be used to study the spinal connections of the different brain stem nuclei. The differences in the axon reaction probably depend on the distance of the lesion from the cell body.

Electrical stimulation.—Malmfors observed (149) but a slight decrease in the fluorescence of the varicosities of the iris nerve net after stimulation of the cervical sympathetic trunk of normal rats. After administration of amine synthesis inhibitors such as H 22/54 and stimulation as above, the fluorescence of most terminals disappeared. On the other hand, Dahlström et al. (46) reported a marked decrease in the fluorescence of the norepinephrine and 5-HT stores of the varicose terminals in the spinal cord after stimulation of the medulla oblongata in normal animals. Obviously, the length and the mode of stimulation affect the degree of depletion.

Effects of drugs.—Authoritative reviews have been published recently on this subject (23, 25, 40). After total depletion by reserpine, the catecholamine fluorescence rapidly reappears in the cell bodies of both central and peripheral adrenergic neurons; a distinct fluorescence is demonstrable around the cell nucleus a few hours after the injection, and the fluorescence is very intense in the whole perikaryon a day later (40, 44, 158). However, the amines in the terminals become demonstrable only after several days. This effect of reserpine has been explained by long-lasting inhibition of the granular uptake of amines; this prevents recovery in the terminals, while the rapid reappearance of amines in the cell body is supposed to be due to formation of new granules in it (44). This is also the main evidence presented for the view that all neuronal catecholamines are bound in granules (23). Electron microscopic studies on the perikaryon are needed before this view can be finally accepted. After depletion with tetrabenazine, the recovery of the amine fluorescence is rapid both in the cell body and in the terminal, presumably because this drug blocks the granular uptake mechanism only for a short time.

All parts of all catecholamine-containing neurons recover their catecholamine content quickly after displacement of the endogenous amines by *m*-tyrosine (40, 44). After administration of α-methyl-*m*-tyrosine, the recovery in the terminals of some neurons is rapid, in those of others slow. The slowly recovering cells have been concluded to be norepinephrine neurons, in which α-methyl-*m*-tyrosine is converted into metaraminol, which for a long time blocks the norepinephrine uptake. DOPAmine neurons recover rapidly because such oxidation does not occur. This differential response to α-methyl-*m*-tyrosine has been used to discriminate histochemically between noradrenergic and DOPAminergic neurons in the central nervous system (25, 40, 91). After administration of α-methyl-dihydroxyphenylalanine, reserpine fails to

produce disappearance of amine fluorescence, presumably because α-methylnorepinephrine, which gives the fluorescence reaction, replaces endogenous norepinephrine and is resistant to reserpine (24).

Amine uptake in vivo.—Hillarp & Malmfors (118, 149) demonstrated that norepinephrine injected into the lingual vein or directly in the eye is rapidly taken up by the preterminal and terminal nerve fibers of the iris. This uptake through the axon membrane resulted in an increased fluorescence along the whole axon. It was inhibited by cocaine but not by reserpine and it was very transient unless monoaminoxidase had been inhibited by nialamide. The slower uptake in the synaptic varicosities is apparently due to active uptake by granular vesicles, which is inhibited by reserpine.

Nonterminal axons, normally difficult to demonstrate by fluorescence microscopy, can be made fluorescent by increasing experimentally their amine content (23, 25, 40, 91, 149, 158). Interesting differences have been found in the amine uptake between catecholamine- and 5-HT-containing neurons. The latter have been found to become intensely fluorescent throughout after treatment with a monoaminoxidase inhibitor alone, both in normal and in reserpinized animals. Because of the blood-brain barrier, administration of both dihydroxyphenylalanine and monoaminoxidase inhibitor fails to cause such fluorescence in the central DOPAmine or norepinephrine neurons of reserpinized animals except for the area postrema, where such a barrier is lacking (92). It is therefore possible to demonstrate the 5-HT neurons selectively by first depleting all amines with reserpine and subsequently administering nialamide, which restitutes the 5-HT neurons (40).

Amine uptake in vitro.—Angelakos & King (5, 9) were the first to study histochemically the amine uptake of the adrenergic tissues *in vitro*. They incubated isolated preparations of rat iris in Tyrode's solution with DOPAmine, norepinephrine, or epinephrine. In each case, a distinct increase was observed in the catecholamine fluorescence of the nerve fibers. The presence of adenosine mono-, di-, or triphosphate was necessary but not the presence of glucose.

Experiments by Eränkö & Räisänen (73) furnished somewhat different results: incubation with norepinephrine in Ringer's solution caused but a slight increase in the intensity of the formaldehyde-induced fluorescence of the iris, whether adenosine triphosphate was present or not. On the other hand, incubation with tyramine, DOPAmine, epinephrine, or 5-HT caused a marked decrease in the amine fluorescence, which was readily restituted to normal by subsequent incubation in 1 μg/ml of norepinephrine. This restitution occurred in the absence of adenosine triphosphate, but the presence of glucose was essential. In accordance with our incubation experiments, Angelakos et al. (6) observed a drastic depletion of norepinephrine in an isolated atrium of the guinea pig heart after perfusion with epinephrine.

Since injected catecholamines do not penetrate the blood-brain barrier, except for certain brain regions such as the area postrema (92, 94), it is usually not possible to study the amine uptake of the central adrenergic

neurons of living animals. Hamberger & Masuoka (113) have incubated brain slices with monoamines and observed such an uptake *in vitro*. After an experimental lesion of the blood-brain barrier, amine uptake by the brain cells has been histochemically demonstrated in living animals (106).

Monoamines and electron microscopic granules.—The pioneer studies by Von Euler and associates (78, 79) clearly showed more than ten years ago that catecholamines are highly concentrated in granules prepared from homogenates of nervous tissue. Subsequently, numerous electron microscopic studies on sympathetic synapses revealed, in addition to the small "empty" vesicles which are normally present in the synapses, other vesicles containing an electron-dense core (49, 54, 103, 143, 167, 186, 187). Several observations indicate that such granular vesicles are carriers of synaptic monoamines: (*a*) fixatives and stains employed in electron microscopy form electron-dense compounds with catecholamines (194); (*b*) the granular vesicles are found in regions rich in aminergic nerve fibers (49, 102, 143, 169, 170, 177, 194); (*c*) sympathetic denervation is associated with a loss of both amines and granular vesicles (48, 102, 165); (*d*) amine depletion after administration of drugs is accompanied by a loss of the granular vesicles (36, 48, 152, 165, 176); (*e*) monoaminoxidase inhibitors which prevent such depletion also prevent the loss of granular vesicles (36, 48); (*f*) after constriction of an adrenergic nerve, both catecholamines and granular vesicles accumulate above the lesion (16, 127); (*g*) administration of amines increases the number of granular vesicles (165); and (*h*) catecholamines are taken up by the granular vesicles in the sympathetic nerves, as was elegantly shown by Wolfe et al. (195), using autoradiographic localization of tritiated norepinephrine at the electron microscopic level.

Other pools of amines.—Several other observations suggest that the granular vesicles may not be the only ultrastructural storage element of transmitter amines: (*a*) granular vesicles have not been found in all such synapses which are aminergic (120, 155); (*b*) there are always agranular vesicles in synapses which contain granular ones [e.g. (48, 49, 54, 102, 143, 167, 169, 170, 186)]; (*c*) efforts to isolate pure fractions of granular vesicles have been unsuccessful, and vesicle fractions with a high norepinephrine content have but a low percentage of granular vesicles (168, 194); (*d*) increase in norepinephrine concentration due to administration of monominoxidase inhibitors is not always accompanied by an increase in the number of granular vesicles (36); (*e*) the proportion of granular vesicles does not increase when such fractions are induced to take up norepinephrine *in vitro* (194); (*f*) vesicles obtained from catecholamine-poor regions of the brain may be shown to possess dense cores when suitably prepared (193, 194); and (*g*) the number of granular vesicles is too small in the intersynaptic parts of terminal nerve fibers and in the perikaryons of the adrenergic neurons to account for their intense formaldehyde-induced fluorescence (142).

These objections certainly deserve further examination. However, many failures to demonstrate granular vesicles may be due to inadequate tech-

niques. This has been emphasized by two successful investigators, De Robertis (48, 101) who considers fixation by perfusion necessary in studying central synapses, and Richardson (171) who has recently found that capriciousness in demonstrating the granular vesicles can be avoided by using 3 per cent potassium permanganate as a fixative. Even these authors have always found the smaller "empty" vesicles with the granular ones.

New histochemical amine methods.—Several combination methods have recently been proposed suitable for the histochemical demonstration of catecholamines at the ultrastructural level: (*a*) formaldehyde and ammoniacal silver (60); (*b*) formaldehyde and osmium tetroxide (13, 48, 101); (*c*) glutaraldehyde and potassium dichromate (15, 196); (*d*) glutaraldehyde and osmium tetroxide (35); (*e*) glutaraldehyde and ammoniacal silver (190). The methods have provided satisfactory results with the adrenal medulla, but most of them still await application to nervous structures. However, Bloom & Barrnett (15) and Wood (196) have recently reported promising results with glutaraldehyde and potassium dichromate in demonstrating the dense-core vesicles in adrenergic nerve endings. Preliminary experiments with formaldehyde vapor fixation for electron microscopy suggest that diffusible pools of catecholamines are easily lost by fixation in aqueous solutions (75).

To conclude, the evidence now available indicates that granular vesicles are an important but probably not the only structure for monoamine storage. It is of interest that Kopin (137) has presented a hypothetical model of a nerve ending which incorporates, in addition to granules, a membranous structure, which is necessary to explain some biochemical and pharmacological observations. Finally, Von Euler (78) has emphasized the potential importance of reversible binding of amines by membrane phospholipids.

Cholinesterases

Development of methods.—In 1949, Koelle & Friedenwald (134) described a method for histochemical demonstration of cholinesterase activity in tissue sections. The method was subsequently improved by Koelle (130) to eliminate diffusion artifacts, and the new method was used to study the distribution of cholinesterases in peripheral (132) and central (131) nervous structures. The method has since then been extensively used, as such or in a slightly modified form. Acetyl- and butyrylthiocholine serve as substrates in the original method and its many modifications. Since copper and sulfate ions are present in the incubation medium, enzymatically liberated thiocholine is precipitated as copper thiocholine sulfate, a colorless, poorly soluble compound, whose distribution can be examined as such by phase contrast microscopy, as proposed by Holmstedt (121), or after treatment with sulfide solution to render the precipitate brown (130).

Discrimination between esterases.—Specific inhibitors play an important role in histochemical esterase techniques. Acetylthiocholine is readily split not only by acetylcholinesterase but also by nonspecific cholinesterase, and the latter must be inhibited by a selective inhibitor. The specificity problem

and the use of the inhibitors have recently been discussed by several authors (50, 100, 122, 133, 135, 164). Substrates other than thiocholine esters have also been used (20, 69, 115, 123, 136, 164).

Acetylcholinesterase as a marker of cholinergic neurons.—Acetylcholinesterase has been found to be a very consistent enzyme in being present selectively in nervous structures and specifically in the cell bodies, axons, and terminals of all cholinergic neurons, while nonspecific cholinesterase is present predominantly in glial cells, and in some neurons and in non-nervous structures as well (115, 133, 189). There is a fairly good correlation between acetylcholinesterase activity, cholinacetylase activity, and acetylcholine (ACh) content in the brain, as was shown already by Feldberg & Vogt (88) and recently by Lewis, Shute & Silver (148) [see also (117, 133, 154)]. However, Shute & Lewis (180, 181) have emphasized that the cell bodies of many neurons exhibit acetylcholinesterase activity, although their axons are devoid of cholinesterase and cholinacetylase activity, which proves them noncholinergic; the presence of acetylcholinesterase on the axon membrane is the essential histochemical criterion of a cholinergic neuron (146). The best criterion of a cholinergic neuron is of course the presence of high concentrations of ACh in its terminal. In view of Whittaker's (194) recent electron microscopic observations on ACh-containing synaptic vesicles, histochemical demonstration of ACh does not seem impossible. For the time being, an intense acetylcholinesterase activity can nevertheless be considered a tentative suggestion of the cholinergic nature of a neuron (125).

Axon reaction.—After division of the axon, acetylcholinesterase activity of the associated cell body decreases at the same time that the activity in the proximal stump of the divided nerve increases (115, 145, 185). Härkönen (115) examined the superior cervical ganglion of the rat after division of the postganglionic nerves near the ganglion. This resulted in an almost complete disappearance of the acetylcholinesterase activity, not only from the ganglion cells but also from the preganglionic fibers and synapses. Less marked changes in the activities of other enzymes and a complete restitution indicated that the changes were not due to interference with the blood supply of the ganglion. A distinct decrease in the acetylcholinesterase activity was seen in the ventral horn cells of the spinal cord after division of the sciatic nerve by Söderholm (185) and a similar decrease in the hypoglossal nucleus was observed after division of the hypoglossal nerve by Lewis & Shute (145). Use has been made of the piling up of acetylcholinesterase activity in the severed axons to trace their origin in the central nervous system (180).

Cholinergic pathways in the brain.—Previous work on the brain with the cholinesterase method has been reviewed in several articles (131, 133, 138, 180, 181, 185). Therefore, only some newer ones are mentioned here. Krnjevic & Silver (138, 139) carried out a thorough study on the distribution of acetylcholinesterase in the cerebral cortex of the cat. A previously unknown tangential system of fine fibers was observed, the terminal network of which was closely related to deep pyramidal cells. Since many of these cells are readily

excitable with ACh, it was concluded that the acetylcholinesterase-containing tangential system provided cholinergic innervation for these cells. Many fibers of this system probably belong to medial and lateral projections from the basal corpus striatum and the septal region. This again has been suggested by Shute & Lewis (179) to be the forebrain extension of the midbrain reticular formation. Thus, the tangential cortical system of acetylcholinesterase-positive fibers may be a final link in the reticular ascending, activating pathway, which is fully consistent with the pharmacology of cortical arousal.

Distribution of acetylcholinesterase in the hippocampal region of the rat was studied by Mathisen & Blackstad (151) and by Shute & Lewis (181) who, in another paper (180), studied the cholinesterase-containing pathways of the hindbrain. In all these studies, use was made of experimental lesions to control the validity of the histochemical observations.

Blood-brain barrier and cholinesterases.—In many species, the brain capillaries exhibit an intense nonspecific cholinesterase activity. Joo & Csillik (126) have recently observed that cholinesterase activity is lacking from such areas of the rat brain, e.g., the area postrema, from which the blood-brain barrier is known to be absent. It was concluded that the barrier function is correlated to the cholinesterase activity, which was electron microscopically localized in pinocytotic vesicles of the vascular endothelium in the barrier-protected brain areas.

Peripheral acetylcholinesterase-positive structures.—Earlier studies have been admirably described in a recent review (133). Other reviews include a symposium publication (175) and three monographs, one by Gerebtzoff (97) on cholinesterases and two others by Zacks (197) and Csillik (33) on the muscle end plate. Cholinesterases have also been thoroughly examined in recent histochemical papers on the retina (76), the superior cervical ganglion (115), and the spinal cord (185). Some other recent observations are mentioned here.

Biscoe & Silver (11) studied the cat carotid body and found both acetylcholinesterase and nonspecific cholinesterase in nerve fibers around the glomus cells, which themselves were nonreactive. Division of the preganglionic trunk to the superior cervical ganglion or of the sinus nerve had no effect on these fibers, but cutting of the postganglionic nerve resulted in their disappearance. The observations strongly suggest cholinergic sympathetic innervation of the glomus cells, and electron microscopic observations have indeed demonstrated typical synapses on them (12). Fibers exhibiting both types of cholinesterase activity were reported by Palkama (163) also in the carotid body of the rabbit.

Large variations in the acetylcholinesterase activity of individual cell bodies in the spinal ganglion of the rat were observed by Kokko (136). She quantitated by microscopic cytophotometry the acetylcholinesterase activity and compared it with the activity of some other enzymes in the same cells by examination of the neighboring sections. A significant positive correlation was thus found between the acetylcholinesterase and acid phosphatase activities in individual cells. Both of these enzymes were negatively

correlated to the cell size. Giacobini (98) determined quantitatively the cholinesterase activity of individual spinal ganglion cells which were separated by microdissection. He reported two populations of cells, one exhibiting an intense, the other a weak, cholinesterase activity. The former was considered truly cholinergic.

Electron microscopic methods.—While Koelle's (130) thiocholine method is admirable for light microscopic demonstration of cholinesterases, its localizing power is insufficient for electron microscopic studies. Barrnett (10) proposed thiolacetic acid for this purpose, with satisfactory results as far as accurate localization was concerned, but lack of specificity was admitted.

Recently, several successful modifications have been reported in which the more specific substrate acetylthiocholine has been employed for electron microscopy. Lewis & Shute (144, 147) described an incubation medium containing, like the Koelle (130) method, copper sulfate for capturing the liberated thiocholine. Karnovsky & Roots (129) incorporated ferricyanide into the substrate mixture; it is reduced by liberated thiocholine, and the enzymatic activity is revealed by a fine precipitate of brown copper ferrocyanide. Koelle & Gromadzki (135) have recently proposed aurous gold as a capturing ion, instead of copper, to obtain a fine precipitate with either thiocholine or thiolacetate, and the preliminary results have been promising.

Electron microscopic observations.—Relatively little work has as yet been done with the electron microscopic cholinesterase methods. However, there is a fair agreement between the results obtained with the different methods. Thus, in the muscle end plate, the acetylcholinesterase activity has been localized in the axon and muscle plasma membranes within the synaptic region by Barrnett (10) with thiolacetic acid and by Lewis & Shute (144) with acetylthiocholine. Pre- and postsynaptic membranes, axon membranes, and the endoplasmic reticulum of the perikaryons of the cholinergic neurons are likewise positive with both substrates, as was observed in the brain of the rat (153, 189), the cockroach (184), and the wood ant (141) with the thiolacetic acid method, and in the rat brain with the thiocholine method (144) and its copper ferrocyanide modification (178). This modification has also been observed to stain the axon membranes of all nonmyelinated fibers in the sciatic nerve (174) and in the salivary gland (96).

The synaptic vesicles have been reported to give a positive reaction toward thiolacetic acid in the muscle end plate (10) and the nervous synapses (141, 153, 181, 184), but not toward acetylthiocholine either in the muscle end plate (144) or in the nervous synapses (178, 181). However, vesicles exhibiting a positive acetylcholinesterase reaction with the copper ferrocyanide modification of the thiocholine method have been observed inside the sciatic axons (174).

Correlation of Catecholamines and Cholinesterases

Sympathetic ganglia.—Early studies on the acetylcholinesterase activity in the sympathetic ganglia revealed marked differences in the intensity of individual ganglion cells, which ranged from strong to weak [see (133)]. The

strongly positive ones were proposed by Koelle (132) to be cholinergic sympathetic cells, and confirmatory evidence of this assumption in the cat was presented by Sjöqvist (182). However, in the ganglia of many other species the majority of cells, necessarily including many adrenergic cones, exhibit a moderate or intense acetylcholinesterase activity (132). The problem became especially interesting when Burn & Rand (21, 22) presented the hypothesis that the release of norepinephrine from adrenergic fibers is mediated by ACh. When subsequent work showed that also the catecholamine fluorescence of individual cells of sympathetic ganglia is very variable (65, 110), it was of obvious interest to correlate the acetylcholinesterase activity and the amine content in individual ganglion cells.

Modification of an earlier method by Eränkö (59) proved successful for this purpose: a short low-temperature exposure to formaldehyde vapor of frozen-dried ganglia made norepinephrine sufficiently fluorescent for photomicrography, while the acetylcholinesterase activity was preserved and subsequently demonstrated in the same section (61, 62). Jacobowitz & Koelle (124, 125) independently presented a similar method, applied to cryostat sections of fresh tissue. Hamberger & Norberg (107) proposed the use of two neighboring cryostat sections for the same purpose.

In several sympathetic ganglia of the cat, Hamberger, Norberg & Sjöqvist (110, 111) observed that the majority of cells exhibited a weak acetylcholinesterase reaction but an intense catecholamine fluorescence, while the minority showed a weak fluorescence but an intense acetylcholinesterase activity. The results were concluded to indicate the presence of distinct adrenergic and cholinergic neurons in the ganglia. Entirely different results were obtained by Eränkö & Härkönen (68) in the superior cervical ganglion of the rat: weak, moderate, or strong norepinephrine fluorescence of individual ganglion cells was indiscriminately associated with weak, moderate, or strong acetylcholinesterase activity. The high amine content and the intense acetylcholinesterase activity in some of the cells were taken to suggest that these cells are at the same time both adrenergic and cholinergic, which fits in well with the Burn & Rand (21, 22) hypothesis. However, the cytoplasmic cholinesterase of these cells may be taken to reflect their cholinoceptive rather than cholinergic nature (181). Furthermore, it is of interest, although somewhat confusing, that nonspecific cholinesterase has been shown by Härkönen (115) to be inversely correlated with the intensity of the catecholamine fluorescence in the superior cervical ganglion of the rat, in the same way as acetylcholinesterase is correlated in the cat (110).

Peripheral sympathetic fibers.—The same problem has been approached by studying catecholamines and acetylcholinesterase in peripheral sympathetic nerve fibers. Eränkö, Härkönen & Räisänen (66, 72) observed that there were many fine fibers in the nerve net of the dilator muscle in the rat iris which exhibited both catecholamine fluorescence and acetylcholinesterase activity, while other fibers were found to contain either catecholamine or acetylcholinesterase but not both. It was tentatively concluded that the

fibers containing both catecholamine and acetylcholinesterase originated from similar cells of the superior cervical ganglion. However, the reservation was expressly made that catecholamine and acetylcholinesterase may be in closely concomitant fibers which cannot be resolved by light microscopy. Our observations were confirmed by Ehinger & Falck (53).

In an independent study, Jacobowitz & Koelle (125) observed, likewise, fibers containing both catecholamine and acetylcholinesterase in the vas deferens of the guinea pig, as well as in the uterus and the tube of the cat. Such fibers could not be found in the vas deferens or in the nictitating membrane of the cat, and their presence remained uncertain in the same organ of the rabbit. The findings were considered compatible with a restricted version of the Burn & Rand hypothesis, according to which liberation of ACh may cause subsequent release of norepinephrine from the same or adjacent fiber.

Denervation experiments.—Csillik & Koelle (34) observed that removal of the superior cervical ganglion of the rat caused a rapid disappearance of all the fluorescent fibers from the iris and also a slower degeneration and disappearance of some 15 to 20 per cent of the acetylcholinesterase positive fibers. These fibers were assumed to originate from those cell bodies in the superior cervical ganglion which possess a moderate or high acetylcholinesterase activity. In a similar study, Ehinger & Falck (53) failed to observe any appreciable reduction in the number of acetylcholinesterase positive fibers after excision of the cervical sympathetic chain. On the other hand, a considerable reduction in the number of acetylcholinesterase-containing fibers was observed after removal of ciliary ganglion, although no overt reduction of adrenergic nerves was detected.

All the above cited authors agree concerning several important points: (*a*) some sympathetic fibers contain catecholamine but not acetylcholinesterase; (*b*) other fibers contain acetylcholinesterase but not catecholamine; (*c*) fibers which appear single in the light microscope, but may in fact enclose several axons, contain both acetylcholinesterase and catecholamine; (*d*) liberation of catecholamines through a cholinergic link is possible through either of the structural mechanisms given in (*c*).

Electron microscopic clues.—Terminal sympathetic axons are known to run close together enclosed in the same Schwann's sheath (170). Therefore, electron microscopic studies are imperative before it can be confirmed or rejected that an axon may be at the same time adrenergic and cholinergic.

Since "empty" nongranular vesicles can always be demonstrated in adrenergic synapses, in addition to granular vesicles (48, 102, 169, 170), and since relatively pure fractions of such "empty" vesicles contain high concentrations of ACh (48, 194), it is possible throughout that adrenergic synapses also contain ACh. However, the empty vesicles of the adrenergic synapses may serve some other function. More reliable criteria are therefore necessary.

Intense acetylcholinesterase activity of the axon membrane has been proposed as a reliable criterion of a cholinergic neuron (180, 181). Looking for the presence or absence of such an activity in axons known to contain

catecholamine can therefore be expected to provide valuable information. Preliminary electron histochemical studies in our laboratory (unpublished) suggest indeed the presence of high acetylcholinesterase activity in the terminal adrenergic axons of the rat iris.

However, final solution of the problem can be expected only when ACh and catecholamine can be demonstrated at the ultrastructural level in a single synapse. In view of the rapid development of electron histochemical techniques, the solution may be found in the not too distant future.

LITERATURE CITED

1. Amin, A. H., Crawford, T. B. B., and Gaddum, J. H., *J. Physiol. (London)*, **126**, 596 (1954)
2. Anden, N.-E., Carlsson, A., and Hillarp, N.-Å., *Acta Pharmacol. Toxicol.*, **21**, 183 (1964)
3. Anden, N.-E., Dahlström, A., Fuxe, K., and Larsson, K., *Am. J. Anat.*, **116**, 329 (1965)
4. Anden, N.-E., Norberg, K.-A., Olson, L., *Acta Physiol. Scand.*, **66**, 501 (1966)
5. Angelakos, E. T., *Science*, **145**, 503 (1964)
6. Angelakos, E. T., Bloomquist, E. J., and King, M., *Life Sci.*, **4**, 1515 (1965)
7. Angelakos, E. T., Fuxe, K., and Torchiana, M. L., *Acta Physiol. Scand.*, **59**, 184 (1963)
8. Angelakos, E. T., Glassman, P. M., Millard, R. W., and King, M., *Comp. Biochem. Physiol.*, **15**, 313 (1965)
9. Angelakos, E. T., and King, M., *J. Histochem. Cytochem.*, **13**, 282 (1965)
10. Barrnett, R. J., *J. Cell Biol.*, **12**, 247 (1962)
11. Biscoe, T. J., and Silver, A., *J. Physiol. (London)*, **179**, 8P (1965)
12. Biscoe, T. J., and Stehbens, W. E., *Nature*, **208**, 708 (1965)
13. Benedeczky, I., Puppi, A., and Tigyi, A., *Acta Biol. Acad. Sci. Hung.*, **15**, 271 (1965)
14. Bertler, Å., Falck, B., and Owman, C., *Acta Physiol. Scand., Suppl. 239*, **63** (1964)
15. Bloom, F. E., and Barrnett, R. J., *Nature*, **210**, 599 (1966)
16. Blümcke, S., and Niedorf, H. R., *Z. Zellforsch. Mikroskop. Anat.*, **68**, 724 (1965)
17. Brundin, J., *Acta Physiol. Scand., Suppl. 259*, **66** (1965)
18. Brundin, J., and Wirsen, C., *Acta Physiol. Scand.*, **61**, 203 (1964)
19. Brundin, J., and Wirsen, C., *Acta Physiol. Scand.*, **61**, 505 (1964)
20. Burstone, M. S., *Enzyme Histochemistry* (Academic Press, New York and London, 621 pp., 1962)
21. Burn, J. H., and Rand, M. J., *Nature*, **194**, 163 (1959)
22. Burn, J. H., and Rand, M. J., *Ann. Rev. Pharmacol.*, **5**, 163 (1965)
23. Carlsson, A., *Pharmacol. Rev.*, **18**, 541 (1966)
24. Carlsson, A., Dahlström, A., Fuxe, K., and Hillarp, N.-Å., *Acta Pharmacol. Toxicol.*, **22**, 270 (1965)
25. Carlsson, A., Falck, B., and Hillarp, N.-Å., *Acta Physiol. Scand., Suppl. 196*, **56** (1962)
26. Carlsson, A., Falck, B., Hillarp, N.-Å., and Torp, A., *Acta Physiol. Scand.*, **54**, 385 (1962)
27. Corrodi, H., and Hillarp, N.-Å., *Helv. Chim. Acta*, **46**, 2425 (1963)
28. Corrodi, H., and Hillarp, N.-Å., *Helv. Chim. Acta*, **47**, 911 (1964)
29. Corrodi, H., Hillarp, N.-Å., and Jonsson, G., *J. Histochem. Cytochem.*, **12**, 582 (1964)
30. Corrodi, H., and Jonsson, G., *J. Histochem. Cytochem.*, **13**, 484 (1965)
31. Corrodi, H., and Jonsson, G., *Acta Histochem.*, **22**, 247 (1965)
32. Costa, E., Revzin, A. M., Kuntzman, R., Spector, S., and Broche, B. B., *Science*, **133**, 1822 (1961)
33. Csillik, B., *Functional Structure of the Post-Synaptic Membrane in the Myoneural Junction* (Academic Publishing House, Budapest, 1965)
34. Csillik, B., and Koelle, B. G., *Acta Histochem.*, **22**, 350 (1965)
35. Coupland, R. E., and Hopwood, D., *J. Anat.*, **100**, 227 (1966)
36. Clementi, F., *Experientia*, **21**, 171 (1965)
37. Dahl, E., Falck, B., von Mecklenburg,

C., and Myhrberg, H., *Gen. Comp. Endocrinol.*, **3**, 693 (1963)
38. Dahl, E., Falck, B., von Mecklenburg, C., and Myhrberg, H., *Quart. J. Microscop. Sci.*, **104**, 531 (1963)
39. Dahlström, A., *J. Anat.*, **99**, 677 (1965)
40. Dahlström, A., and Fuxe, K., *Acta Physiol. Scand., Suppl. 232*, **62** (1964)
41. Dahlström, A., and Fuxe, K., *Z. Zellforsch. Mikroskop. Anat.*, **62**, 602 (1964)
42. Dahlström, A., and Fuxe, K., *Acta Physiol. Scand., Suppl. 247*, **64** (1965)
43. Dahlström, A., and Fuxe, K., *Acta Oto-Laryngol.*, **59**, 65 (1965)
44. Dahlström, A., Fuxe, K., and Hillarp, N.-Å., *Acta Pharmacol. Toxicol.*, **22**, 277 (1965)
45. Dahlström, A., Fuxe, K., Hökfelt, T., and Norberg, K.-A., *Acta Physiol. Scand.*, **66**, 507 (1966)
46. Dahlström, A., Fuxe, K., Kernell, D., and Sedvall, G., *Life Sci.*, **4**, 1207 (1965)
47. Dahlström, A., and Zetterström, B. E. M., *Science*, **147**, 1583 (1965)
48. De Robertis, E., *Pharmacol. Rev.*, **18**, 413 (1966)
49. De Robertis, E., and Pellegrino de Iraldi, A., *J. Biophys. Biochem. Cytol.*, **10**, 361 (1961)
50. Diegenbach, P. C., *Nature*, **207**, 308 (1965)
51. Ehinger, B., *Acta Physiol. Scand., Suppl. 268*, **67** (1966)
52. Ehinger, B., *Z. Zellforsch. Mikroskop. Anat.*, **71**, 146 (1966)
53. Ehinger, B., and Falck, B., *Life Sci.*, **4**, 2097 (1965)
54. Elfvin, L.-G., *J. Ultrastruct. Res.*, **8**, 441 (1963)
55. Eränkö, O., *Acta Anat., Suppl. 17*, **16** (1952)
56. Eränkö, O., *Acta Anat.*, **22**, 331 (1954)
57. Eränkö, O., *Acta Endocrinol.*, **18**, 174 (1955)
58. Eränkö, O., *Studies on the Adrenal Medulla* (Doctoral thesis, Univ. Edinburgh, Scotland, 1958)
59. Eränkö, O., *Histochemie*, **1**, 257 (1959)
60. Eränkö, O., *Ciba Found. Symp. Adrenergic Mechanisms*, 103–8 (Wolstenholme, G. E. W., and O'Connor, M., Eds., Churchill, London, 1960)
61. Eränkö, O., *J. Histochem. Cytochem.*, **12**, 487 (1964)
62. Eränkö, O., *Pharmacol. Rev.*, **18**, 353 (1966)
63. Eränkö, O., *Proc. Roy. Soc. (London)* (In press, 1966)
64. Eränkö, O., (Personal communication, 1961) [Acknowledged by Falck, Hillarp, Thieme and Torp (82)]
65. Eränkö, O., and Härkönen, M., *Acta Physiol. Scand.*, **58**, 285 (1963)
66. Eränkö, O., and Härkönen, M., *Acta Physiol. Scand.*, **61**, 299 (1964)
67. Eränkö, O., and Härkönen, M., *Acta Physiol. Scand.*, **63**, 411 (1965)
68. Eränkö, O., and Härkönen, M., *Acta Physiol. Scand.*, **63**, 511 (1965)
69. Eränkö, O., Härkönen, M., Kokko, A., and Räisänen, L., *J. Histochem. Cytochem.*, **12**, 570 (1964)
70. Eränkö, O., and Penttilä, O. (Unpublished observations, 1965)
71. Eränkö, O., and Räisänen, L., *J. Histochem. Cytochem.*, **9**, 54 (1961)
72. Eränkö, O., and Räisänen, L., *Acta Physiol. Scand.*, **63**, 505 (1965)
73. Eränkö, O., and Räisänen, L., in *Mechanisms of Release of Biogenic Amines*, 73 (Euler, U. S. von, Rosell, S., and Uvnäs, B., Eds., Pergamon Press, Oxford, 1966)
74. Eränkö, O., and Räisänen, L., *J. Histochem. Cytochem.* (In press)
75. Eränkö, O., Räisänen, L., and Hänninen, L. (Unpublished observations, 1966)
76. Esilä, R., *Acta Ophthalmol., Suppl. 77* (1963)
77. Euler, U. S. Von, *Pharmacol. Rev.*, **18**, 29 (1966)
78. Euler, U. S. Von, in *Mechanisms of Release of Biogenic Amines*, 211 (Euler, U. S. von, Rosell, S., and Uvnäs, B., Eds., Pergamon Press, Oxford, 1966)
79. Euler, U. S. Von, and Hillarp, N.-Å., *Nature*, **177**, 44 (1956)
80. Falck, B., *Acta Physiol. Scand., Suppl. 197*, **56** (1962)
81. Falck, B., Häggendahl, J., and Owman, C., *Quart. J. Exptl. Physiol.*, **48**, 253 (1963)
82. Falck, B., Hillarp, N.-Å., Thieme, G., and Torp, A., *J. Histochem. Cytochem.*, **10**, 348 (1962)
83. Falck, B., and Owman, C., in *Mechanisms of Biogenic Amines*, 59 (Euler, U. S. von, Rosell, S., and Uvnäs, B., Eds., Pergamon Press, Oxford, 482 pp., 1966)
84. Falck, B., and Owman, C., *Acta Univ. Lund., Sect. II*, No. 7 (1965)
85. Falck, B., and Rorsman, H., *Experientia*, **19**, 205 (1963)
86. Falck, B., and Torp, A., *Med. Exptl.*, **5**, 429 (1961)

87. Falck, B., and Torp, A., *Med. Exptl.*, **6**, 169 (1962)
88. Feldberg, W., and Vogt, M., *J. Physiol. (London)*, **107**, 372 (1948)
89. Frontali, N., and Norberg, K.-A., *Acta Physiol. Scand.*, **66**, 243 (1966)
90. Fuxe, K., *Acta Physiol. Scand., Suppl. 247*, **64**, IV (1965)
91. Fuxe, K., *Evidence for the Existence of Monoamine Neurons in the Central Nervous System* (M.D. thesis, Karolinska Institutet, Stockholm, 1965)
92. Fuxe, K., and Hillarp, N.-Å., *Life Sci.*, **3**, 1403 (1964)
93. Fuxe, K., and Nilsson, B. Y., *Experientia*, **21**, 641 (1965)
94. Fuxe, K., and Owman, C., *J. Comp. Neurol.*, **125**, 337 (1965)
95. Fuxe, K., and Sedvall, G., *Acta Physiol. Scand.*, **64**, 75 (1965)
96. Garrett, J. R., *J. Roy. Microscop. Soc.*, **85**, 135 (1965)
97. Gerebtzoff, M. A., *Cholinesterases* (Pergamon Press, London, New York, Paris, and Los Angeles, 1959)
98. Giacobini, E., *Acta Physiol. Scand.*, **45**, 238 (1959)
99. Gillespie, J. S., and Kirpekar, S. M., *J. Physiol. (London)*, **179**, 46P (1965)
100. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **68**, 354 (1948)
101. Gonzalez, A., and De Robertis, E., *Neurology*, **13**, 758 (1963)
102. Grillo, M. A., *Pharmacol. Rev.*, **18**, 387 (1966)
103. Grillo, M. A., and Palay, S. L., in *Intern. Congr. Electron Microscopy, 5th, Philadelphia*, **2**, U-1 (Breese, S. S., Ed., Academic Press, New York, 1962)
104. Häggendal, I., and Malmfors, T., *Acta Physiol. Scand.*, **64**, 58 (1965)
105. Håkanson, R., and Owman, C., *J. Neurochem.*, **12**, 417 (1965)
106. Hamberger, A., and Hamberger, B., *Z. Zellforsch. Mikroskop. Anat.*, **70**, 386 (1966)
107. Hamberger, B., and Norberg, K.-A., *J. Histochem.*, **12**, 48 (1964)
108. Hamberger, B., and Norberg, K.-A., *Acta Physiol. Scand.*, **65**, 235 (1965)
109. Hamberger, B., and Norberg, K.-A., *Intern. J. Neuropharmacol.*, **4**, 41 (1965)
110. Hamberger, B., Norberg, K.-A., and Sjöqvist, F., *Life Sci.*, **9**, 659 (1963)
111. Hamberger, B., Norberg, K.-A., and Sjöqvist, F., *Proc. Intern. Pharmacol. Meeting, 2nd, Prague, 1963*, 41 (1964)
112. Hamberger, B., Norberg, K.-A., and Ungerstedt, U., *Acta Physiol. Scand.*, **64**, 285 (1965)
113. Hamberger, B., and Masuoka, D., *Acta Pharmacol. Toxicol.*, **22**, 363 (1965)
114. Hamberger, B., Malmfors, T., and Sachs, C. *J. Histochem. Cytochem.*, **13**, 147 (1965)
115. Härkönen, M., *Acta Physiol. Scand., Suppl. 237*, **63** (1964)
116. Harreveld, A. Van, and Crowell, J., *Anat. Record*, **149**, 381 (1964)
117. Hebb, C. O., in Cholinesterases and anticholinesterase agents, *Heffter-Heubner Handbook Experimental Pharmacology, Suppl. 15*, 55–88 (Koelle, G. B. Ed., Springer-Verlag, Heidelberg, 1963)
118. Hillarp, N.-Å., and Malmfors, T., *Life Sci.*, **3**, 703 (1964)
119. Hökfelt, T., *J. Histochem. Cytochem.*, **13**, 518 (1965)
120. Hökfelt, T., and Nilsson, O., *J. Ultrastruct. Res.*, **12**, 237 (1965)
121. Holmstedt, B., *Acta Physiol. Scand.*, **40**, 331 (1957)
122. Holmstedt, B., and Sjöqvist, F., *Bibliogr. Anat.*, **2**, 1 (1961)
123. Holt, S. J., in *General Cytochemical Methods*, 375–98 (Danielli, J. F., Ed., Academic Press, New York and London, 1958)
124. Jacobowitz, D., and Koelle, G. B., *Pharmacologist*, **5**, 270 (1963)
125. Jacobowitz, D., and Koelle, G. B., *J. Pharmacol. Exptl. Therap.*, **148**, 225 (1965)
126. Joo, F., and Csillik, B., *Exptl. Brain Res.*, **1**, 147 (1966)
127. Kapeller, K., and Mayor, D., *J. Anat.*, **100**, 439 (1966)
128. Kapeller, K., and Mayor, D., *J. Physiol. (London)*, **182**, 44P (1965)
129. Karnovsky, M. J., and Roots, L., *J. Histochem. Cytochem.*, **12**, 1219 (1964)
130. Koelle, G. B., *J. Pharmacol. Exptl. Therap.*, **103**, 153 (1951)
131. Koelle, G. B., *J. Comp. Neurol.*, **100**, 211 (1954)
132. Koelle, G. B., *J. Pharmacol. Exptl. Therap.*, **114**, 167 (1955)
133. Koelle, G. B., in Cholinesterases and anticholinesterase agents, *Heffter-Heubner Handbook Experimental Pharmacology, Suppl. 15*, 189–298 (Koelle, G. B., Ed., Springer-Verlag, Heidelberg, 1963)
134. Koelle, G. B., and Friedenwald, J. S.,

Proc. Soc. Exptl. Biol. Med., **70**, 617 (1949)
135. Koelle, G. B., and Gromadzki, C. G., *J. Histochem. Cytochem.*, **14**, 443 (1966)
136. Kokko, A., *Acta Physiol. Scand., Suppl. 261*, **66** (1965)
137. Kopin, I. J., in *Mechanisms of Release of Biogenic Amines*, 229–46 (Euler, U. S. von, Rosell, S., and Uvnäs, B., Eds., Pergamon Press, Oxford, 482 pp., 1966)
138. Krnjevic, K., and Silver, A., *J. Anat.*, **99**, 711 (1965)
139. Krnjevic, K., and Silver, A., *J. Anat.*, **100**, 63 (1966)
140. Lagunoff, D., Phillips, M., and Benditt, E. V., *J. Histochem. Cytochem.*, **9**, 534 (1961)
141. Landolt, A. M., and Sandri, C., *Z. Zellforsch. Mikroskop. Anat.*, **69**, 246 (1966)
142. Lenn, N. J., *Anat. Record*, **153**, 399 (1965)
143. Lever, J. D., and Esterhuizen, A. C., *Nature*, **192**, 566 (1961)
144. Lewis, P. R., and Shute, C. C. D., *J. Physiol. (London)*, **175**, 5-7P (1964)
145. Lewis, P. R., and Shute, C. C. D., *J. Physiol. (London)*, **178**, 25-26P (1965)
146. Lewis, P. R., and Shute, C. C. D., *J. Physiol. (London)*, **180**, 8-10P (1965)
147. Lewis, P. R., and Shute, C. C. D., *J. Cell Sci.* (In press)
148. Lewis, P. R., Shute, C. C. D., and Silver, A., *J. Physiol. (London)*, **171**, 65P (1964)
149. Malmfors, T., *Acta Physiol. Scand., Suppl. 248*, **64** (1965)
150. Malmfors, T., and Sachs, C., *Acta Physiol. Scand.*, **64**, 377 (1965)
151. Mathisen, J. S., and Blackstad, T. W., *Acta Anat.*, **56**, 216 (1964)
152. Matsuoka, M., Ishii, S., Shimizu, N., and Imaizumi, R., *Experientia*, **21**, 121 (1965)
153. Mori, S., Maeda, T., and Shimizu, N., *Histochemie*, **4**, 65 (1964)
154. Nachmansohn, D., in Cholinesterases and anticholinesterase agents, *Heffter-Heubner Handbook Experimental Pharmacology, Suppl. 15*, 40–54 (Koelle, G. B., Ed., Springer-Verlag, Heidelberg, 1963)
155. Nilsson, O., *Z. Zellforsch. Mikroskop. Anat.*, **64**, 166 (1964)
156. Norberg, K.-A., *Intern. J. Neuropharmacol.*, **3**, 379 (1964)
157. Norberg, K.-A., *The Sympathetic Adrenergic Neuron and Certain Adrenergic Mechanisms* (M.D. thesis, Karolinska Institutet, Stockholm, 1965)
158. Norberg, K.-A., and Hamberger, B., *Acta Physiol. Scand., Suppl. 238*, **63**, (1964)
159. Norberg, K.-A., and Olson, L., *Acta Physiol. Scand.*, **68**, 183 (1965)
160. Owman, C., *Acta Physiol. Scand., Suppl. 240*, **63** (1964)
161. Owman, C., *Intern. J. Neuropharmacol.*, **2**, 105 (1964)
162. Owman, C., and Sjöstrand, N. O., *Z. Zellforsch. Mikroskop. Anat.*, **66**, 300 (1965)
163. Palkama, A., *Ann. Med. Exptl. Biol. Fenniae (Helsinki)*, **43**, 260 (1965)
164. Pearse, A. G. E., *Histochemistry Theoretical and Applied* (Churchill, London, 997 pp., 1960)
165. Pellegrino de Iraldi, A., and De Robertis, E., *Intern. J. Neuropharmacol.*, **2**, 231 (1964)
166. Penttilä, O., *Ann. Med. Exptl. Biol. Fenniae (Helsinki), Suppl. 9*, **44** (1966)
167. Pick, J., *J. Comp. Neurol.*, **120**, 409 (1963)
168. Potter, L. T., *Pharmacol. Rev.*, **18**, 439 (1966)
169. Richardson, K. C., *J. Anat.*, **96**, 427 (1962)
170. Richardson, K. C., *Am. J. Anat.*, **114**, 173 (1964)
171. Richardson, K. C., *Nature*, **210**, 756 (1966)
172. Ritzén, M., *Rept. Scand. Congr. Cell Res., 4th, June 2–3, 1965*, 62
173. Ritzén, M., (Personal communication in Lund, 1965)
174. Schlaepfer, W. W., and Torack, R. M., *J. Histochem. Cytochem.*, **14**, 369 (1966)
175. Schwarzacher, H. G., Ed., Histochemistry of cholinesterase, Symp., Basel, 1960, *Bibliogr. Anal.*, 2 (1961)
176. Shimizu, N., and Ishii, S., *Arch. Histol. Japan.*, **24**, 489 (1964)
177. Shimizu, N., and Ishii, S., *Z. Zellforsch. Mikroskop. Anat.*, **64**, 462 (1964)
178. Shimizu, N., and Ishii, S., *Histochemie*, **6**, 24 (1966)
179. Shute, C. C. D., and Lewis, P. R., *Nature*, **199**, 1160 (1963)
180. Shute, C. C. D., and Lewis, P. R., *Nature*, **205**, 242 (1965)
181. Shute, C. C. D., and Lewis, P. R., *Z. Zellforsch. Mikroskop. Anat.*, **69**, 334 (1966)
182. Sjöqvist, F., *Acta Physiol. Scand.*, **57**,

339 (1963)
183. Sjöstrand, N. O., *Acta Physiol. Scand., Suppl. 257*, **65** (1965)
184. Smith, D. S., and Treherne, J. E., *J. Cell Biol.*, **26**, 445 (1965)
185. Söderholm, U., *Acta Physiol. Scand., Suppl. 256*, **65** (1965)
186. Szentágothai, J., *Acta Neuroveget. (Vienna)*, **26**, 338 (1964)
187. Taxi, J., *Acta Neuroveget. (Vienna)*, **26**, 360 (1964)
188. Thieme, G., *J. Histochem. Cytochem.*, **13**, 386 (1965)
189. Torack, R. M., and Barrnett, R. J., *Exptl. Neurol.*, **6**, 224 (1962)
190. Tramezzani, J. H., Chiocchio, S., and Wassermann, G. F., *J. Histochem. Cytochem.*, **12**, 890 (1964)
191. Vanov, S., and Vogt, M., *J. Physiol. (London)*, **168**, 939 (1963)
192. Vogt, M., *J. Physiol. (London)*, **123**, 451 (1954)
193. Whittaker, V. P., and Sheridan, M. N., *J. Neurochem.*, **12**, 363 (1965)
194. Whittaker, V. P., *Pharmacol. Rev.*, **18**, 401 (1966)
195. Wolfe, D. E., Potter, L. T., Richardson, K. C., and Axelrod, J., *Science*, **138**, 440 (1962)
196. Wood, J. G., *Nature*, **209**, 1131 (1966)
197. Zacks, S., *The Motor Endplate* (Saunders, Philadelphia, Pa., and London, 321 pp., 1964)

Histochemie 8, 369—376 (1967)

ELECTRON MICROSCOPIC DEMONSTRATION OF CHOLINESTERASES IN NERVOUS TISSUE

O. Eränkö, L. Rechardt and L. Hänninen
Department of Anatomy, University of Helsinki,
Siltavuorenpenger, Helsinki 17, Finland

Received December 1, 1966

Summary. Acetylcholinesterase was demonstrated at ultrastructural level in the motor nerve cells of rat's spinal cord using the Karnovsky-Roots modification of Koelle's thiocholine method. Selective inhibitors were employed to check the validity of the reaction.

Prolonged formaldehyde fixation improved the poor penetration of the reactive agents and diminished the relatively large crystal size of the end product, which were the two main difficulties of the method. The preservation of ultrastructure was highly improved, when thin sections were made without freezing using a tissue chopper.

Acetylcholinesterase was localized in the nuclear envelope, in the rough-surfaced endoplasmic reticulum, in medium-sized vesicles of the Golgi apparatus, and around synaptic terminals. Synaptic vesicles were found negative.

Histochemical localization of cholinesterases has been much studied since the publication by Koelle (1951) of an acetylthiocholine method, which is excellent for light microscopy but not as such suitable for electron microscopic studies because of the large crystal size of the resulting precipitate. Methods using other substrates, notably thiolacetic acid, have provided better localization, but their specificity is questionable (Barrnett, 1962; Torack and Barrnett, 1962; Mori, Maeda and Shimizu, 1964; Koelle and Foroglou-Kerameos, 1965).

Therefore, several efforts have been made to improve the localizing power of the thiocholine method. Birks and Brown (1960) proposed silver instead of copper as a capturing metal. Using copper, as in Koelle's method, Lewis and Shute (1964, 1966) and Shute and Lewis (1966) increased the efficiency of the capture reaction by adjustments in the composition of the incubation medium.

Karnovsky and Roots (1964) introduced a "direct-coloring" modification of the thiocholine method, in which enzymatically liberated thiocholine reduces ferricyanide into ferrocyanide and a brown precipitate of copper ferrocyanide is formed. This method combines the specificity of thiocholine as a substrate with direct colour formation at the sites of enzymatic activity.

After preliminary trials with all the above methods, we subjected the last-mentioned technique to a more detailed study, using motor ventral horn cells of the rat's spinal cord as material. The present report describes our experiences of the conditions under which satisfactory results can be obtained.

Methods

Both formaldehyde and glutaraldehyde were used as fixatives. Formaldehyde was employed as a 4% solution either with 1% $CaCl_2$ or with 0.3 M sucrose in 0.075 M phosphate buffer, pH 7.4. The fixation time varied from 4 hours to 2 days. Glutaraldehyde was employed in a concentration of 2.5%, and it was buffered to pH 7.2 with 0.1 M cacodylate buffer.

The fixation time was 2—4 hours. The fresh tissues were always immersed in an ice-cold fixative, and thereafter the fixation was continued in a refrigerator at about 5° C.

After fixation, the tissues were rinsed in the same solution in which the fixative was dissolved. Formalin-fixed tissues were rinsed from 2 hours to overnight, glutaraldehyde-fixed tissues always overnight in the refrigerator. Pieces of fixed and washed tissue were sectioned without freezing with a McIlwain tissue chopper at 50 μ (Smith and Farquhar, 1963), or with a freezing microtome at 10—20 μ.

All sections were handled free-floating. They were preincubated for 20 minutes in the substrate-free incubation medium with one of the inhibitors employed, *i.e.* 10^{-6} M tetra-isopropylpyrophosphoramide (iso-OMPA), 10^{-5} M 1.5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (284C51) or 10^{-5} M eserine. The enzyme reaction was then started by adding the substrate in a few drops of maleate buffer. The final concentration of acetylthiocholine iodide was 0.2 mM. The effect of acidity was studied by varying the pH between 4.5 and 7.5, adjusting the concentration of sodium citrate accordingly (Karnovsky and Roots, 1964).

In some experiments 0.3 M sucrose was added into the incubation medium. Incubation was carried out at about 5° C, 23° C or 37° C, and the incubation time ranged from 15 minutes to 2 hours.

The incubation was terminated when a faint brown colour was observed in the section under the light microscope. This took 15—60 minutes depending on the temperature. The sections were then rinsed in 4 changes of ice-cold 0.3 M sucrose solution, about 5 minutes each. The lateral nerve cell group of the ventral horn was then prepared under a dissecting microscope. This minute piece of tissue, which contained several motor nerve cells, was post-fixed for one hour in 1% osmium tetroxide solution buffered at pH 7.6 with a veronal-acetate buffer (Rhodin, 1954; Zetterquist, 1956). After washing, dehydration and embedding in Epon, sections were cut at about 600 Å with an LKB Ultrotome and counterstained with lead citrate (Reynolds, 1963). For electron micrography, Akashi Tronscope and Siemens Elmiskop I were used.

Results

Sufficient fixation was found to be of outmost importance, not only for cytological preservation but also to obtain a sharp localization of the histochemical reaction. Freezing always resulted in poor preservation. Fig. 1 shows a section fixed for two hours in the glutaraldehyde mixture and subsequently sectioned with freezing microtome. Gross ice-crystal artifacts have destroyed many details. Frozen sections from pieces fixed with formaldehyde were equally unsatisfactory.

For this reason freezing was omitted altogether, and the tissue pieces were sectioned fresh with the McIlwain tissue chopper. The results were thus much improved. However, after 4 hours' fixation in 2.5% glutaraldehyde and subsequent washing overnight, the reaction was not very clearly localized in any cell organelle, except for the nuclear membrane, and there was a wide pericellular space indicating poor tissue preservation. Essentially similar results were obtained even if fixation lasted overnight.

The results were distinctly improved by prolonged fixation. Fixation for 48 hours in the formaldehyde-sucrose fixative at 4° C resulted both in good preservation of tissue and in sharp localization of the reaction product in the cytoplasmic organelles. In addition to the nuclear membrane (Fig. 2), some cytoplasmic membranes exhibited a positive reaction as well, notably the coarse granulated endoplasmic reticulum (Fig. 3). A positive reaction was also found in certain areas of the Golgi apparatus (Fig. 4). Although quite acceptable results were often obtained after fixation by immersion of small fresh pieces of the spinal cord in the fixative, the pericellular structures tended to become loosened

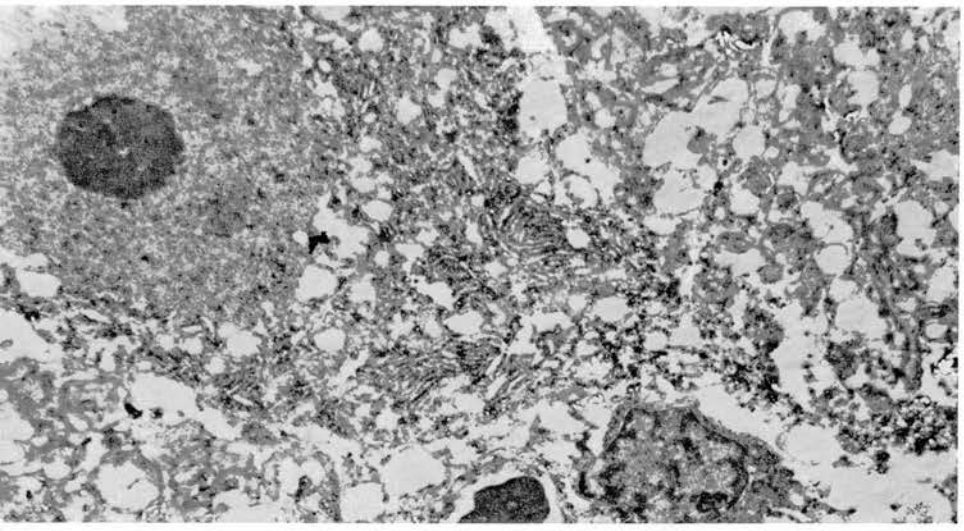
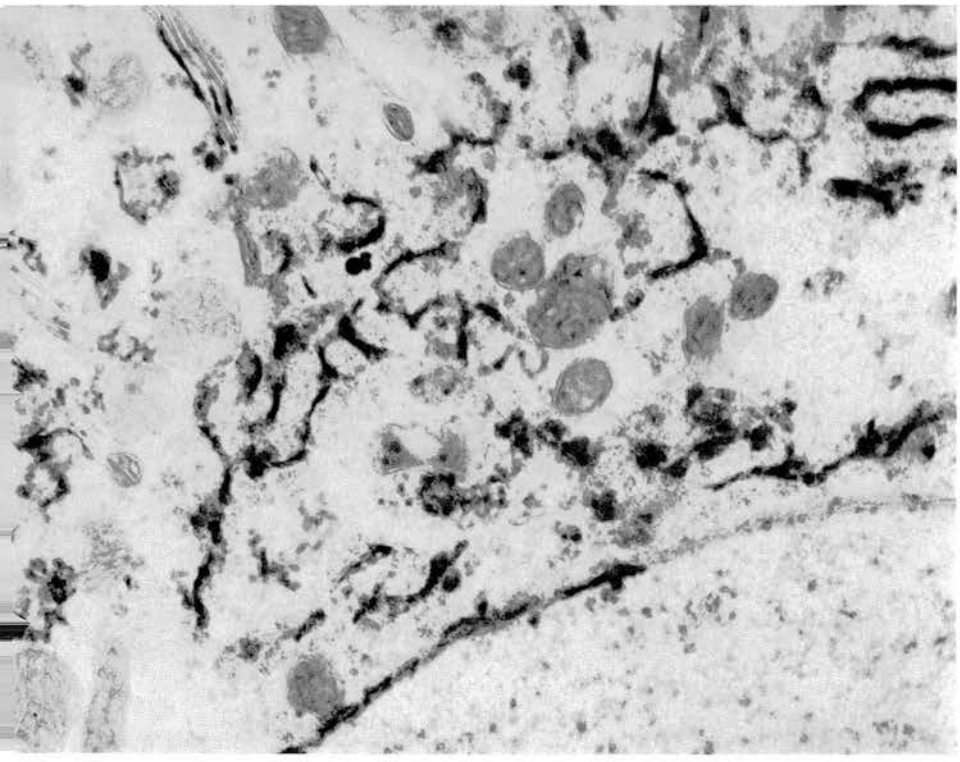


Fig. 1. Cholinesterase reaction in a section fixed for 2 hours in 2.5% glutaraldehyde buffered to pH 7.2 with cacodylate buffer. Section cut with a freezing microtome before incubation. Gross ice-crystal artifacts are obvious. ×9000



'ig. 2. Distribution of acetylcholinesterase in a motor nerve cell. Fixation for 48 hours in 4% formaldehyde uffered to pH 7.4 with phosphate buffer. The tissue preservation is much improved. The reaction product is pparent as a discontinuous line both at the nuclear membrane and between the rough-surfaced membranes of the endoplasmic reticulum. ×28200

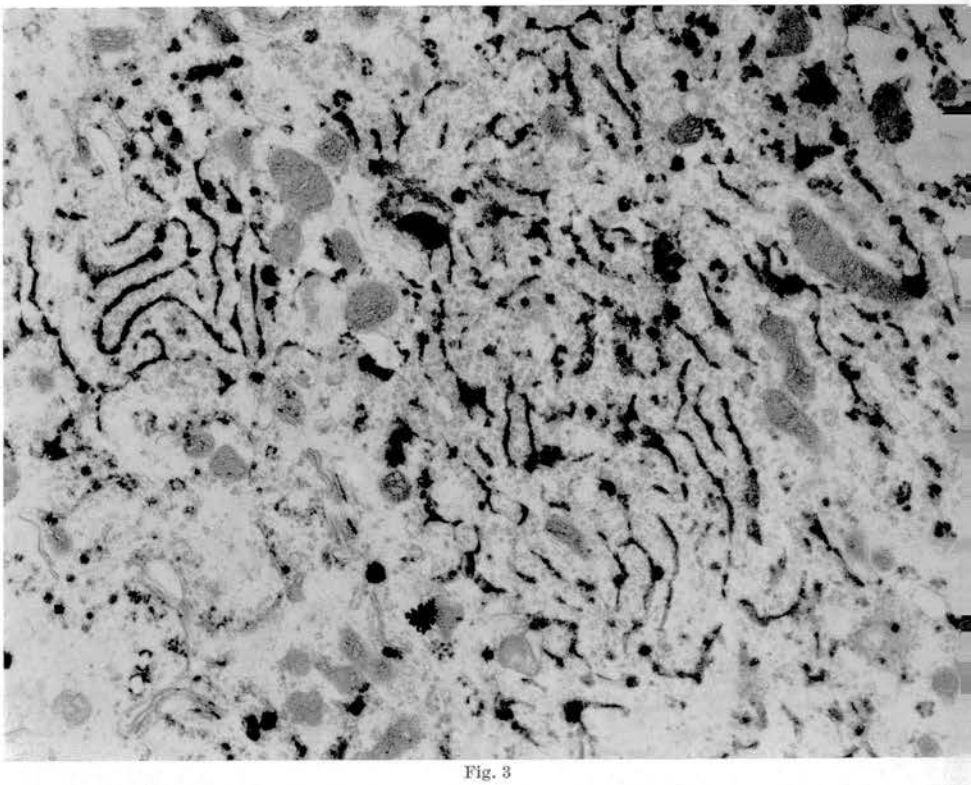


Fig. 3

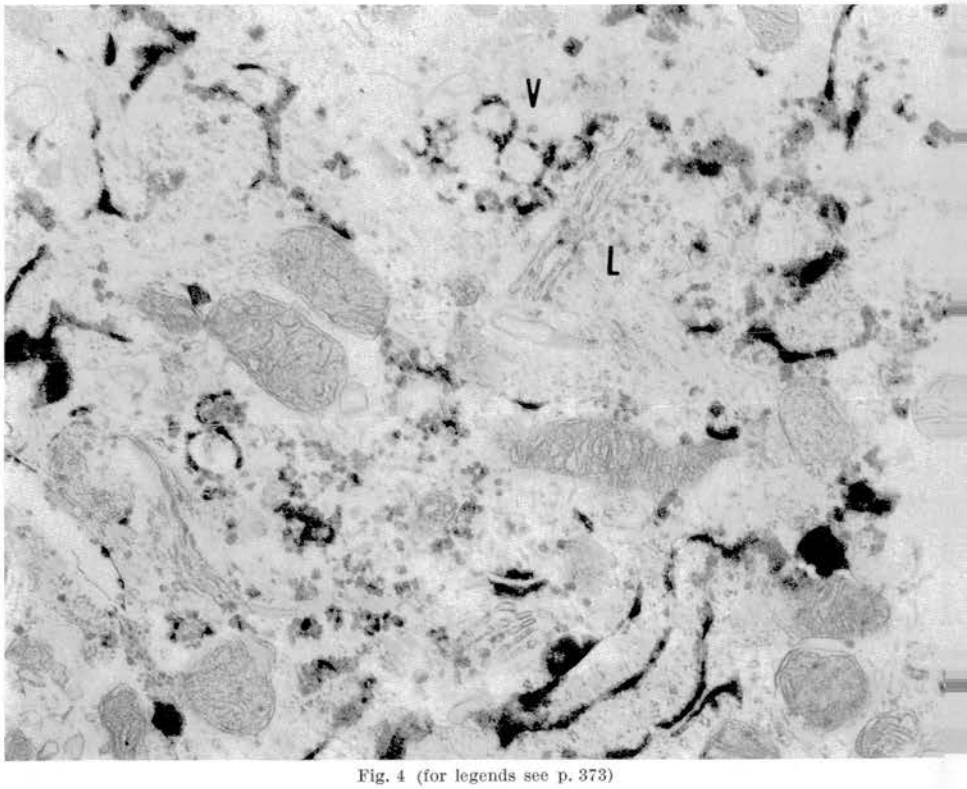


Fig. 4 (for legends see p. 373)

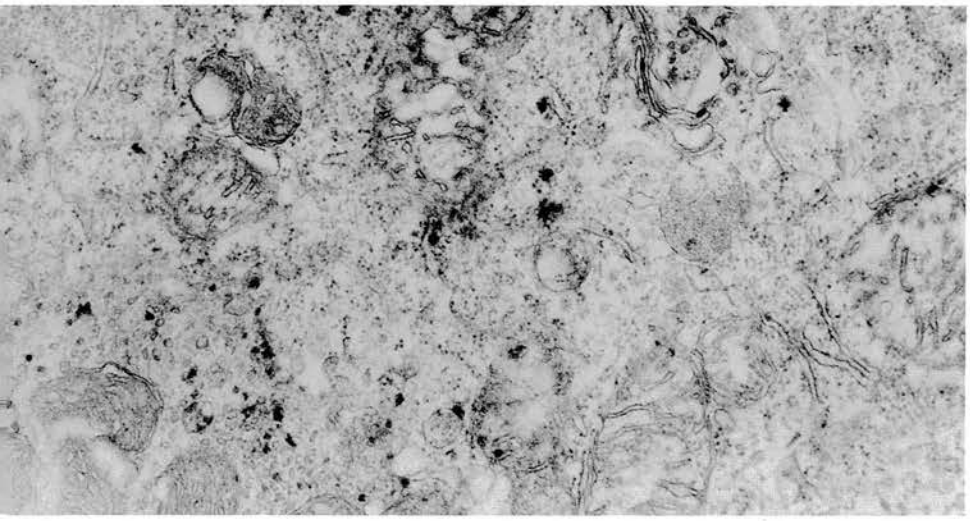


Fig. 5. Almost negative cholinesterase reaction in a motor nerve cell incubated with acetylthiocholine and both 10^{-5} M 284C51 and 10^{-6} M iso-OMPA. There is very little reaction product and it is scattered randomly throughout the cell. ×28200

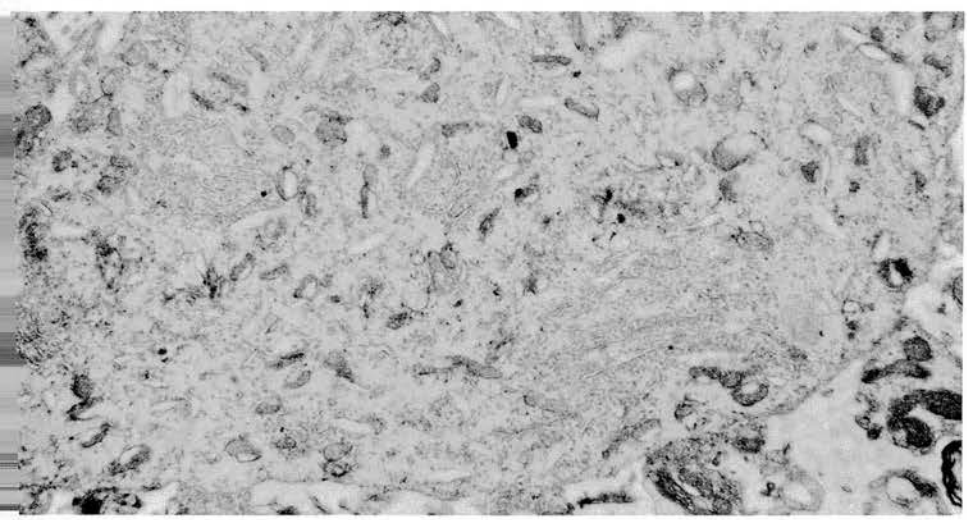


Fig. 6. Essentially negative reaction in a nerve cell incubated with acetylthiocholine and 10^{-5} M eserine. ×12000

even if the cytoplasm of the nerve cell was well preserved. Best results were obtained with pieces fixed as about 200 μ thick slices of fresh tissue.

One hour's incubation at 5° C was found best for both sharp localization of the histochemical reaction and tissue preservation. At this temperature the reaction reached but a very thin zone from the surface, approximately 10 μ on both

Fig. 3. Acetylcholinesterase reaction in the cytoplasm of a motor nerve cell. Fixation for 48 hours in 4% formaldehyde buffered to pH 7.4 with phosphate buffer. Incubation for 1 hour at 5° C with 10^{-6} M iso-OMPA as an inhibitor. The reaction product fills the interspace of rough-surfaced endoplasmic reticulum. ×15000

Fig. 4. Acetylcholinesterase in a motor nerve cell. The section was treated as that in Fig. 3. Parts of the Golgi apparatus contain the reaction product, especially larger vacuoles (*V*), while the lamellae (*L*) are devoid of activity. ×28200

sides of the section. The penetration was better if an incubation medium was used which did not contain sucrose. After preliminary studies, the substrate medium was always adjusted at pH 6.0.

All the above reactions were obtained with acetylthiocholine as a substrate and 10^{-5} M iso-OMPA as a specific inhibitor of non-specific cholinesterase. The reaction was almost negative, when 10^{-5} M 284C51 was employed together 10^{-6} M iso-OMPA (Fig. 5) but a few dark granules were randomly distributed all over the section. Similar results were obtained with 10^{-5} M eserine (Fig. 6), while no precipitate at all was visible, when the substrate was omitted from the incubation mixture (Fig. 7). Thus, the reaction described was due to acetylcholinesterase.

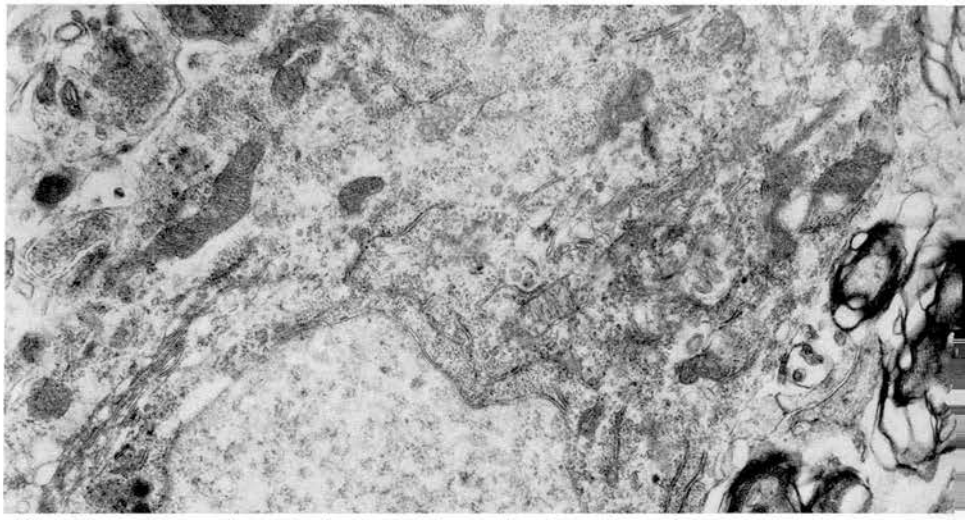


Fig. 7. Nerve cell from a tissue piece incubated in the reaction mixture from which acetylthiocholine was omitted. No reaction product is visible. ×15000

Discussion

In our hands, the use of frozen sections proved quite unsatisfactory for electron microscopic studies. The preservation of ultrastructure was always definitely better, when thin sections of fixed tissue were made without freezing, as was also observed by Lewis and Shute (1966). This procedure, however, diminished the penetration of the histochemical reagents, and the reaction was obtained only at a depth of about 10 μ. That penetration improved, when no sucrose was added into the incubation medium, was recently reported also by Bell (1966) who used the light microscope only.

Glutaraldehyde has been advocated as a specially suitable fixing agent in the electron microscopic study of hydrolytic enzymes. In our hands, it did not show any advantages over formaldehyde in the study of cholinesterases. Glutaraldehyde is indeed known to have an inhibitory effect on cholinesterase activity (Torack and Barrnett, 1962). Apparently because of poor penetration, some areas of the nervous tissue immersed in this fixative were inadequately fixed.

While better results were obtained with formalin, this was only true for pieces fixed for extended periods. Shute and Lewis (1966) have recently proposed

prolonged fixation in formalin after glutaraldehyde for electron microscopic studies of cholinesterases in the brain. Prolongation of formalin fixation also proved to have in our experience a favourable effect on the crystal size of the final precipitate. This may have been due both to better fixation of the tissue and to partial inhibition by formalin of the enzymatic activity, so that the histochemical capture reaction can take place before diffusion of the products of hydrolysis.

Fixation by arterial perfusion of formalin, proposed for electron microscopic studies of the nervous tissue already by GONZALEZ AGUILAR and DE ROBERTIS (1963), has been examined in our preliminary experiments (unpublished). Superior preservation of pericellular structures was observed.

The intracellular localization of the enzymatic activity observed in the present study is as a whole in agreement with recently reported observations on nervous tissues: sympathetic ganglion (KOELLE and FOROGLOU-KERAMEOS, 1965), dorsal nucleus of the vagus (SHIMIZU and ISHII, 1966), and motor neurons of the ventral horn (LEWIS and SHUTE, 1966). However, we observed a positive reaction also in the Golgi apparatus, especially in the medium-sized vesicles. Such a localization has not been reported by previous workers, but it seems natural enough considering the role of the Golgi apparatus in the transport of the products synthetized by the cell. The nucleus, the ribosomes, the lysosomes, the mitochondria and the plasma membrane were found to be negative. The two last mentioned, consistently negative membranous structures indicate that the reaction product does not in a nonspecific way precipitate at all membranes in general. No reaction product was observed in synaptic vesicles either, even if the membrane surrounding the synaptic terminal exhibited a positive reaction.

After the present study was completed, KOELLE and GROMADZKI (1966) reported an improved thiocholine method, using gold instead of copper as the capturing ion. This method was said to combine the high specificity of the thiocholine as a substrate with high localizing power. Their observations at electron microscopic level have not yet been published.

References

BARRNETT, R. J.: The fine structural localization of acetylcholinesterase at the myoneuronal junction. J. Cell. Biol. **12**, 247—262 (1962).

BELL, C.: Use of the direct-coloring thiocholine technique for demonstration of intracellular neuronal cholinesterases. J. Histochem. Cytochem. **14**, 567—570 (1966).

BIRKS, R. I., and L. M. BROWN: A method for locating the cholinesterase of a mammalian myoneuronal junction by electron microscopy. J. Physiol. (Lond.) **152**, 5P—7P (1960).

GONZALES AGUILAR, F., and E. DE ROBERTIS: A formalin-perfusion fixation method for histophysiological study of the central nervous system with the electron microscope. Neurology (Minneap.) **13**, 758—771 (1963).

KARNOVSKY, M. J., and L. ROOTS: A „direct-coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. **12**, 219—221 (1964).

KOELLE, G. B.: The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. J. Pharmacol. exp. Ther. **103**, 153—171 (1951).

—, and C. FOROGLOU-KERAMEOS: Electron microscopic localization of cholinesterases in a sympathetic ganglion by a gold-thiolacetic acid method. Life Sci. **4**, 417—424 (1965).

—, and C. G. GROMADZKI: Comparison of the gold-thiocholine and gold-thiolacetic acid methods for the histochemical localization of acetylcholinesterase and cholinesterases. J. Histochem. Cytochem. **14**, 443—454 (1966).

Lewis, P. R., and C. C. D. Shute: Demonstration of cholinesterase activity with the electron microscope. J. Physiol. (Lond.) **175**, 5P—7P (1964).

— — The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. J. Cell Sci. **1**, 381—390 (1966).

Mori, S., T. Maeda, and N. Shimizu: Electron-microscopic histochemistry of cholinesterases in the rat brain. Histochemie **4**, 65—72 (1964).

Reynolds, E. S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208—212 (1963).

Rhodin, J.: Thesis Stockholm 1954.

Shimizu, N., and S. Ishii: Electron microscopic histochemistry of acetylcholinesterase of rat brain by Karnovsky's method. Histochemie **6**, 24—33 (1966).

Shute, C. C. D., and P. R. Lewis: Electron microscopy of cholinergic terminals and acetylcholinesterase-containing neurones in the hippocampal formation of the rat. Z. Zellforsch. **69**, 334—343 (1966).

Smith, R. E., and M. G. Farquhar: Preparation of thick sections for cytochemistry and electron microscopy by a nonfreezing technique. Nature (Lond.) **200**, 691 (1963).

Torack, R. M., and R. J. Barrnett: Fine structural localization of cholinesterase activity in the rat brain stem. Exp. Neurol. **6**, 224—244 (1962).

Prof. Dr. O. Eränkö
Department of Anatomy
University of Helsinki
Helsinki 17, Siltavuorenpenger 20

Acta physiol. scand. 1967. 69. 255—256

From the Department of Anatomy, University of Helsinki, Finland

Effect of Hydrocortisone Administration In Utero on the Adrenaline and Noradrenaline Content of Extra-Adrenal Chromaffin Tissue in the Rat

By

Olavi Eränkö, Matti Lempinen and Liisa Räisänen

Previous studies have shown that hydrocortisone prevents the normal postnatal degeneration of the para-aortic chromaffin bodies, so-called organ of Zuckerkandl (OZ) (Lempinen 1964). Moreover, it causes the appearance of adrenaline in it, not normally present in the OZ of newborn rats (Eränkö, Lempinen and Räisänen 1966). The present study was designed to find out whether administration of hydrocortisone during prenatal development of the OZ would have an effect on its catecholamine composition.

Hydrocortisone was administered subcutaneously into the rat foetuses 16 days after copulation, *i.e.*, 5 days before the birth, by exposing the uterus of the pregnant rat under ether anesthesia and by injecting 1 mg of hydrocortisone (Hydro-Adreson, Organon) in each foetus. Immediately after birth the foetuses were killed, and the retroperitoneal tissue block containing the OZ was examined chromatographically for catecholamines, as in the previous study (Eränkö *et al.* 1966).

Fig. 1 shows that only the noradrenaline spot is visible in chromatograms obtained from OZ of normal newborn rats while both adrenaline and noradrenaline are present in the OZ of rats injected with hydrocortisone *in utero*. Thus, hydrocortisone brought about the appearance of adrenaline in the OZ during a development period when this amine is never normally seen in it. This observation strongly suggests that enclosure inside the cortical tissue in the adrenal gland significantly promotes the adrenaline synthesis of the chromaffin cells. It fits admirably in with the recent observation (Axelrod 1966) that the level of phenylethanolamine N-methyltransferase in the adrenal medulla is regulated by glucocorticoids secreted by the adrenal cortex.

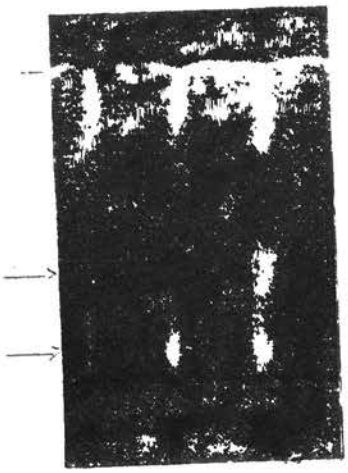


Fig. 1. Fluorescence photograph of a chromatogram developed in phenol-hydrochloric acid for 4 hrs. Natural size.

Left, adrenal cortical tissue; centre, adrenals of 4 normal newborn rats; right, adrenals of 2 newborn rats injected 5 days before birth with 1 mg of hydrocortisone. The upper adrenaline spot is present in the last chromatogram only.

References

Axelrod, J., Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol. Rev.* 1966. *18*. 95—113.

Eränkö, O., M. Lempinen and L. Räisänen, Adrenaline and noradrenaline in the Organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. *Acta physiol. scand.* 1966. *66*. 253—254.

Lempinen, M., Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. *Acta physiol. scand.* 1964. *62*. Suppl. 231.

KUNGL. BOKTR. STHLM 1967

Acta physiol. scand. 1967. 69. 383—384

From the Department of Anatomy, University of Helsinki, Finland

Localization of Noradrenaline and Acetylcholinesterase in the Taenia of the Guinea-pig Caecum

By

GUNNAR ÅBERG[1] and OLAVI ERÄNKÖ

Many studies have been published on physiological and pharmacological responses of the guinea-pig taenia coli, often with controversial results (see Burnstock *et al.* 1966). In the present study, histochemical methods were employed to approach the problem.

Catecholamines were demonstrated using formaldehyde-induced fluorescence Eränkö 1955, Falck, 1962), in the present case noradrenaline is responsible. Koelle's 1951) acetylthiocholine technique together with tetra-isopropylpyrophosphoramide (iso-OMPA) was employed for the demonstration of acetylcholinestrerase activity.

Fig. 1 shows fluorescence in a cross section of the caecum; numerous adrenergic nerve fibres with intensely fluorescent synaptic varicosities are visible in the taenia T), while fewer fibres are present in the circular muscle layer (C). In longitudinal sections of the taenia (Fig. 4), the fluorescent fibres were seen to run parallelly with the smooth muscle fibres, in close proximity to them. These observations are in agreement with those made by Hollands and Vanov (1965).

An abundance of cholinesterase-positive fibres was observed in the taenia (Fig. 2). The smooth muscle cells of the taenia also showed some enzyme activity. The cholinesterase-positive fibres of the circular muscle were less frequent than those in the taenia. Between the muscular layers, the nerve trunks and the ganglion cells of the Auerbach plexus (P) exhibited an intense acetylcholinesterase reaction; cholinergic nerve fibres were observed to emerge from this plexus to the taenia and to the circular muscle.

The Auerbach plexus was also intensely fluorescent (Fig. 1). This fluorescence was due to numerous fluorescent fibres with frequent synaptic varicosities in contact not only with non-fluorescent ganglion cell groups, as observed earlier by Norberg (1964) and Jacobowitz (1965), but probably also with non-fluorescent nerve fibres in the plexus (Fig. 3).

Histochemical examination of "ganglion-free" taenia strip preparations dissected with special care to include only the longitudinal taenia muscle, as is customary for pharmacological experiments, often revealed parts of the Auerbach plexus with ganglion cells surrounded by fluorescent boutons. This observation makes understandable the variable results reported with drugs such as dimethylphenylpiperazinium (see Burnstock *et al.* 1966.)

[1] Present address: Department of Zoophysiology, University of Göteborg, Göteborg, Sweden.

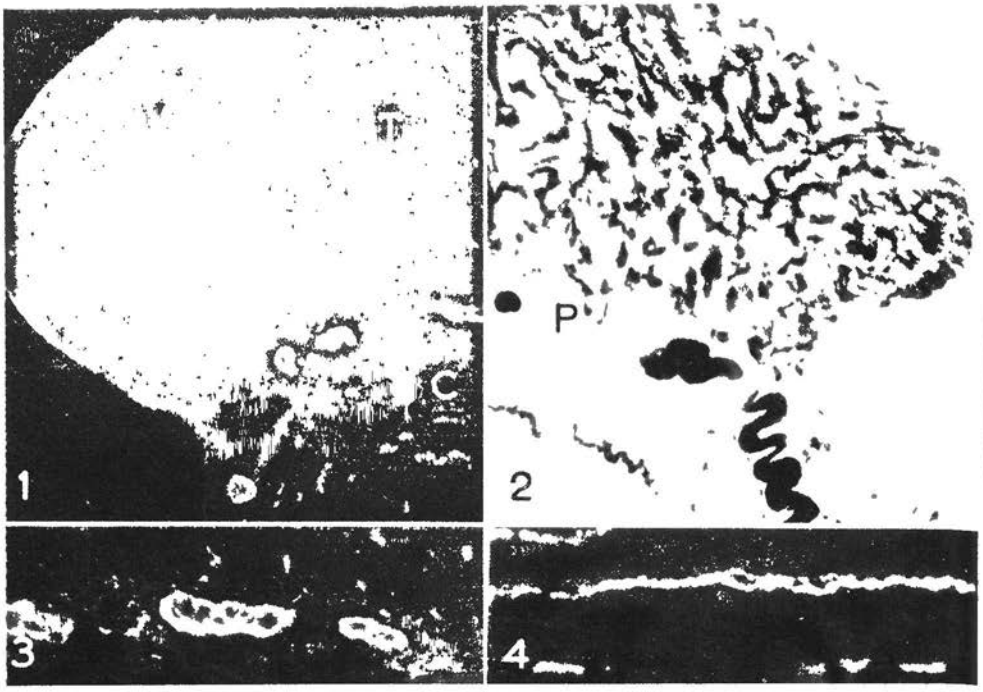


Fig. 1. Fluorescence due to noradrenaline in a cross section of the guinea-pig caecum. T, taenia; C, circular muscle. · 50. Fig. 2. Acetylcholinesterase activity in the guinea-pig caecum. P, site of Auerbach's plexus. · 50. Fig. 3. Detail of the Auerbach plexus. · 100. Fig. 4. Longitudinal section of the taenia. · 100.

Our observations indicate a rich innervation of the guinea-pig taenia, firstly by cholinergic fibres originating from the ganglion cells of the Auerbach plexus and, secondly, by postganglionic adrenergic fibres from sympathetic ganglia outside the gut wall which innervate (a) the ganglion cells, (b) the cholinergic nerve fibres in the Auerbach plexus and (c) the smooth muscle of the taenia. Since all these sites can be influenced by stimulation or drugs, complicated effects can be obtained.

Work supported by research grants from the Finland State Commission of Medical Sciences, the Sigrid Jusélius Foundation and AB Bofors, Mölndal, Sweden.

References

Burnstock, G., G. Campbell and M. J. Rand, The inhibitory innervation of the taenia of the guinea-pig caecum. *J. Physiol. (Lond.)* 1966. *182*. 504—526.

Eränkö, O., Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. *Acta endocrin. (Kbh.)* 1955. *18*. 174—179.

Falck, B., Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.* 1962. *56*. Suppl. 197.

Hollands, B. C. S. and S. Vanov, Localization of catechol amines in visceral organs and ganglia of the rat, guinea-pig and rabbit. *Brit. J. Pharmacol.* 1965. *25*. 307—316.

Jacobowitz, D., Histochemical studies on the autonomic innervation of the gut. *J. Pharmacol. exp. Ther.* 1965. *149*. 358—364.

Koelle, G. B., The elimination of diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol. exp. Ther.* 1951. *103*. 153—17

Norberg, K.-A., Adrenergic innervation of the intestinal wall studied by fluorescence microscop *Int. J. Neuropharmacol.* 1964. *3*. 379—382.

© *Journal of the Royal Microscopical Society, Vol.* 87, *Pt.* 2, *December* 1967. *Pp.* 259–276.
Received 10 *January*, 1967.

The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence

by OLAVI ERÄNKÖ*, *Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki* 17, *Finland*

SYNOPSIS

Catecholamines become intensely fluorescent when exposed to formaldehyde. This is due to closure of the side chain by a methylene bridge and subsequent dehydrogenation, which results in the formation of intensely fluorescent isoquinolines. Exposing freeze-dried tissue to formaldehyde vapour makes it possible to study the distribution of catecholamines by fluorescence microscopy. The present paper deals with the practical performance of the formaldehyde-induced fluorescence.

The technique consists of the following steps:

(1) *Freezing*. This must be rapid. An apparatus is proposed for freezing at maximum rate, in which the tissue slice comes into sudden contact with a cold metal surface.

(2) *Drying*. The tissue must be dried at a temperature below $-40°C$, using an efficient apparatus. The types of apparatus are described and discussed. Small tissue pieces can be dried overnight with an efficient apparatus.

(3) *Formaldehyde condensation*. Formaldehyde is generated by warming paraformaldehyde powder in a glass vessel containing the freeze-dried tissue pieces. The temperature of exposure and the water content of the paraformaldehyde powder must be properly adjusted for each tissue. Exposure for 1 hour at 50°C to paraformaldehyde powder equilibriated with 60 p.c. relative humidity is recommended as a start.

(4) *Embedding, sectioning, and mounting*. The formaldehyde-treated tissue pieces are embedded in paraffin wax or, for better resolution, in epoxy resin. Sections must be spread on the slide without using water. Xylene, paraffin oil, and Entellan are suitable mounting media.

(5) *Fluorescence microscopy*. Any microscope fitted with non-fluorescent optics is suitable for fluorescence work. A sufficiently intense light source is necessary; the high pressure mercury vapour lamp HBO 200 (Osram) is suitable. Special effects can be obtained with different filter combinations. The Schott filters BG 12 and OG 1 are suitable for most practical studies of catecholamines.

* The author wishes to express to the Royal Microscopical Society his sincere appreciation of the honour of being invited to participate in the symposium on "Fluorescence Methods in Histochemistry" in Sheffield, 1966, at which this paper was presented.

Original work pertaining to the contents of this paper has been supported by grants from the Sigrid Jusélius Foundation and the State Commission of Medical Sciences in Finland.

INTRODUCTION

While most histochemical fluorescence-microscopic techniques depend on the use of fluorescent stains or reagents, great interest has recently been focused on a method in which non-fluorescent biogenic amines are rendered intensely fluorescent by treatment with formaldehyde. No doubt condensation with formaldehyde was responsible for much of the apparent autofluorescence previously observed in formalin-fixed sections by many early workers. For instance, Erös (1932) found fluorescence in the so-called yellow cells of the gut, but he thought that autofluorescent lipids were responsible.

A green fluorescence was observed in certain cell groups of the adrenal medulla by the present writer (Eränkö, 1951). It was formaldehyde-induced, *i.e.* seen only in adrenals fixed with formaldehyde solution (Eränkö, 1952). Analysis with specific chemical methods showed subsequently that the medullary cells exhibiting this kind of fluorescence contained a high concentration of noradrenaline. Since it was also found that noradrenaline forms an intensely fluorescence compound with formaldehyde, treatment with formaldehyde was proposed as a histochemical method for noradrenaline (Eränkö, 1955).

Although the fluorescence was stronger in sections dried after exposure, the method was not sufficiently sensitive for the localization of noradrenaline in sympathetic nervous tissue (Eränkö, 1958). However, it has recently been shown (Eränkö & Räisänen, 1966) that a dilute formaldehyde solution made in a Krebs-Ringer solution renders adrenergic nerve fibres intensely fluorescent in the nerve net of the iris. Earlier failures were presumably due to the use of osmotically improperly balanced fluids as solvents for formaldehyde.

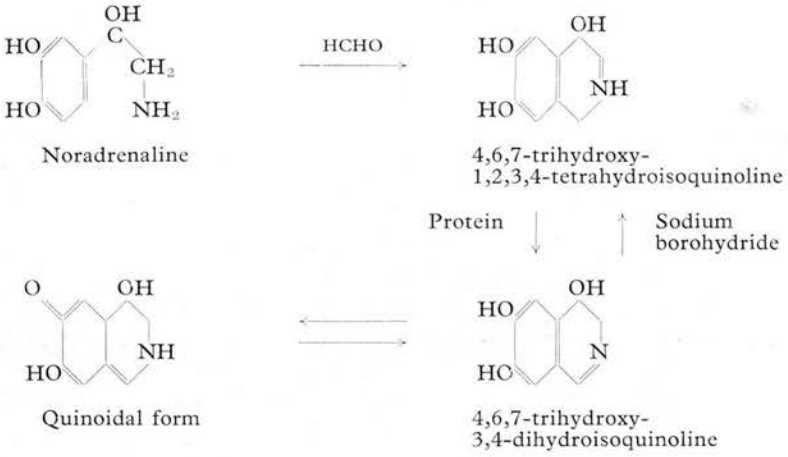
It was independently observed in several laboratories (Eränkö, 1961; Falck & Torp, 1961; Lagunoff *et al.*, 1961) that aromatic monoamines became intensely fluorescent in freeze-dried tissues when they were exposed to formaldehyde vapour. Formaldehyde vapour had previously been used to render the enterochromaffin cells fluorescent, presumably because of their high content of 5-hydroxytryptamine (Barter & Pearse, 1953, 1955). Falck, Hillarp and associates (Carlsson *et al.*, 1962; Falck, 1962; Falck *et al.*, 1962) were the first to report the successful demonstration of catecholamines by formaldehyde vapour in nervous tissues. Since then, a large number of studies have appeared dealing with the distribution of catecholamines and 5-hydroxytryptamine in various tissues. Several reviews have also been published, and the reader mainly interested in the results obtained with the method is referred to them (Acheson, 1965; Euler *et al.*, 1966; Eränkö, 1967).

In the present paper, the emphasis is on the practical aspects, rather than the theory of the method or the results obtained with it. It is preferred to use the non-committal term "formaldehyde-induced fluorescence" (FIF), instead of calling the method after any one investigator since many workers have contributed to its development from the empirical stage into the fairly sensitive and reasonably specific histochemical method which it now is.

THE MECHANISM OF THE REACTION

The FIF method is capable of demonstrating not only catecholamines such as dopamine, noradrenaline, and adrenaline, but also 5-hydroxytryptamine. Moreover, formaldehyde also renders fluorescent the precursors of these compounds, dihydroxyphenylalanine and 5-hydroxytryptophane. The reaction is due to closure of the side chain by a methylene bridge produced from the formaldehyde.

This results in the formation of an isoquinoline or isocarboline derivative, which is not fluorescent but in tissues becomes spontaneously dehydrogenated into an in-



tensely fluorescent compound (Corrodi & Hillarp, 1963, 1964; Corrodi & Jonsson, 1965*a*, *b*). While it has often been emphasized that this reaction occurs only in the presence of dry proteins (*e.g.*, Falck & Owman, 1965), it is also readily demonstrable in spot tests made with pure amine solutions applied to a filter paper which is then allowed to dry (Eränkö, unpublished observations).

The last step can be reversed by treatment with sodium borohydride (Corrodi *et al.*, 1964); the fluorescence disappears but can be regenerated by renewed treatment with formaldehyde vapour. Thus, FIF can be distinguished from non-specific fluorescence due to compounds other than aromatic monamines.

Autofluorescent substances, often present in significant amounts in cell organelles such as lipofuscin granules and lysosomes (Kokko, 1965), are (by definition) intensely fluorescent in tissues not exposed to formaldehyde. However, the intensity of the autofluorescence sometimes increases after treatment with formaldehyde; this is the case in the connective-tissue fibres of human muscle (Lempinen, 1966). Special care is therefore necessary and, whenever possible, chemical analysis should be carried out with specific methods to establish the real nature of the fluorogenic substance. Such an analysis is often desirable also from the point of view of finding out which of the monoamines is involved, even if some conclusions can also be drawn with the aid of other histochemical tests, as will be seen later.

PRACTICAL PERFORMANCE OF THE REACTION

The method is simple in practice and the equipment required need not be very sophisticated. All the steps are, however, somewhat critical, and an error in any one of them ruins the result. Nevertheless strict adherence to simple precautions guarantees reproducible and good results.

The processing of the tissue involves the following steps (1) freezing, (2) drying in a frozen state, (3) exposure to formaldehyde, (4) embedding, sectioning, and mounting, and (5) fluorescence microscopy. The methodological aspects have been discussed in several recent papers (Dahlström & Fuxe, 1964, 1965; Eränkö, 1964; Falck, 1962; Falck *et al.*, 1962; Falck & Owman, 1964; Norberg & Hamberger, 1964; Jacobowitz & Koelle, 1965). In the present paper, each of the steps is considered separately. It must be pointed out that successful demonstration of catecholamines in thin structures, such as the rat iris, is possible without freeze-drying.

Simple stretch preparations, which are allowed to dry at room temperature, can be used (Malmfors, 1965).

(1) *Freezing*

Ice crystals which form during freezing of the tissue are later apparent as empty spaces in the frozen-dried specimen (see, *e.g.*, Pearse, 1960). As the size of the crystals increases when the cooling rate decreases, maximum cooling rate is desirable. Immersion of small tissue pieces (about 3 mm cubes or smaller) in an organic fluid such as isopentane or propane cooled with liquid air or liquid nitrogen is the most commonly adopted method. It provides satisfactory results for the study of catecholamines with the FIF method at low or medium magnification. However, ice crystal artifacts are often manifest at higher magnification in frozen specimens 3 mm thick. It is better, therefore, to freeze slices which are as thin as possible. Devices have been specially designed for cutting such slices from fresh tissue

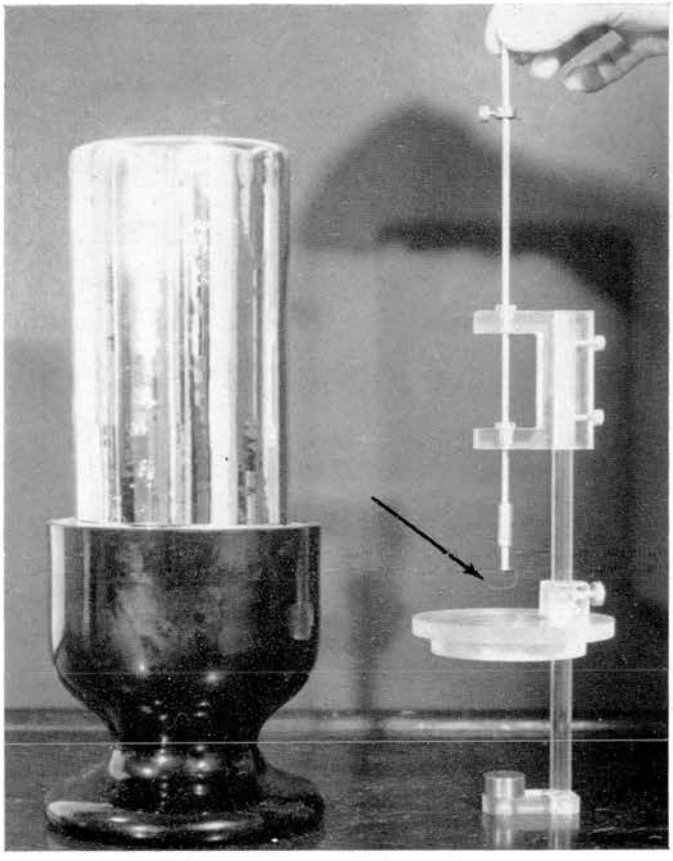


Fig. 1. Apparatus for quick freezing of fresh tissue slices. The slices are spread on the lower surface of the C-shaped copper netting (indicated by the arrow). The copper disc in the lower part of the apparatus is immersed just below the surface of liquid nitrogen contained in the Dewar vessel. Freezing occurs when the rod is allowed to fall. The C-piece prevents squashing of the tissue.

(Majno & Bunker, 1957). The use of small pieces of tissue has the additional advantage of shortening the drying time.

The tissue slice to be frozen is difficult to handle unless it is spread on a metal foil; this is now a common practice. Instead of foil, thin copper mesh, such as the material from which grids are punched for electron microscopy, is practicable. The frozen tissue slice sticks to the mesh and is therefore easy to handle.

For maximum rate of cooling, metal surfaces pre-cooled in liquid nitrogen or helium should be used. Copper disc forceps (Eränkö, 1954) and a falling-piston apparatus (Harreveld & Crowell, 1954) have been designed for the purpose. However, both of these tend to cause distortion by squashing the tissue between the closing surfaces. A device has recently been reported (Eränkö & Kallio, 1967) in which squashing is avoided by using a C-shaped piece of copper netting as a shock-absorber onto which the tissue is attached (fig. 1.).

Care is necessary in handling the tissue before freezing. It is preferable to dissect the pieces to be dried in a cool chamber or on a cooled stage. This not only minimizes post-mortem changes in the tissues, but also increases the rapidity of freezing. Decrease of the tissue temperature from 37°C to, say, −5°C before freezing promotes sudden solidification during subsequent cooling.

Fig. 2. Simple freeze-drying apparatus of the cold finger type, raised from the Dewar vessel containing a cooling mixture. A water-cooled diffusion pump combination is on on the left.

(2) *Drying*

A wide range of freeze-drying devices have been described in the literature (see Pearse, 1960) and many types are commercially available. It is not quite clear which type of apparatus is the best. The present author uses a simple "cold-finger" apparatus (Eränkö, 1954*b*). It is fitted with a liquid nitrogen trap close to the tissue holder and incorporates an oil diffusion pump and a two-stage mechanical pump. This kind of apparatus has the greatest possible water-pumping capacity. Figure 2 illustrates an apparatus in which the outer tube is immersed in a Dewar flask containing diethyl oxalate cooled to $-40°C$ with solid carbon dioxide. Figure 3 shows a similar type of apparatus cooled more conveniently with a refrigerator. Part of the same apparatus is shown in fig. 4 with the outer tube immersed in warm water so as to raise the temperature of the tissue holder (arrow) above the ambient one.

To prevent the tissue temperature from falling too low, the contact between the outer tube of the apparatus and the metal tissue holder must be good, to provide for sufficient heat conduction from outside. Flat-polished surfaces take care of this in the present apparatus. In the basically similar apparatus of Falck and Owman (1965) the tissue holder has been directly moulded onto the bottom of the outer vessel, using a mixture of tin and lead, so as to ensure a perfect fit.

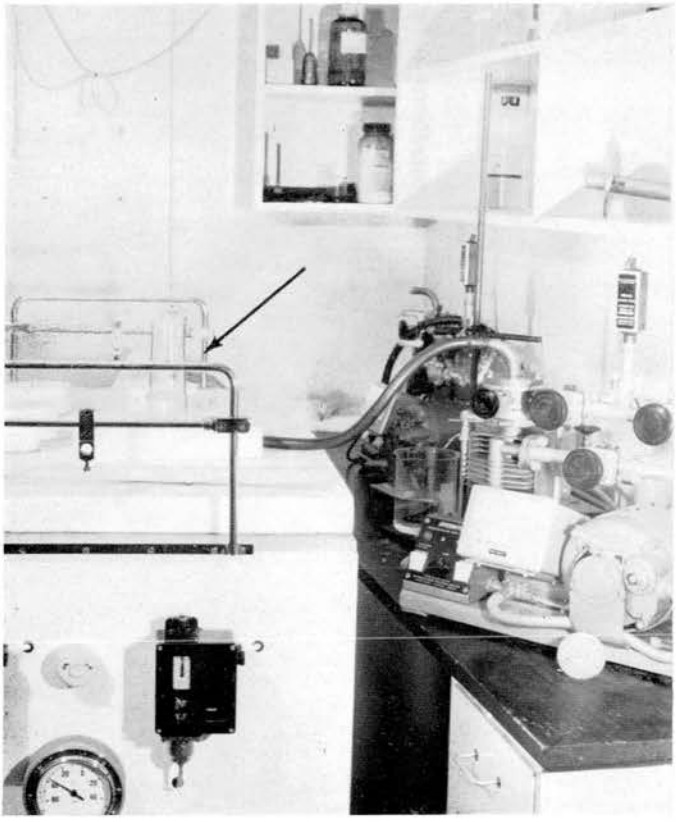


Fig. 3. Freeze-drying apparatus (arrow) in a mechanically-cooled refrigerator. An air-cooled diffusion pump is on the right. The refrigerator can accommodate 4 independently operated freeze-drying devices.

Recently apparatus has been advocated in which a mechanical pump only is used for the production of the vacuum and in which phosphorus pentoxide serves to absorb water vapour. This water trap is placed near a tissue platform cooled either electrically by the Peltier effect (Pearse, 1963), or by a cooling spiral through which cold fluid is pumped (Thieme, 1965). They have been reported efficient for the study of the FIF, a statement which there is no reason to doubt. Thieme (1965) has reported excellent demonstration of the FIF in specimens up to 3 mm thick after drying for 5 hours. However, several laboratories working with the same type of equipment find it necessary to dry for several days, preferably for over a week. Poor contact with the tissue platform and the resulting decrease in the tissue temperature is an important reason for long drying times.

The nature of the tissue is also important. Thus, a large full-thickness piece of the duodenum of the cat can be satisfactorily dried overnight at -40°C in a simple apparatus but the much smaller superior cervical ganglion of the rat takes about a week in the same apparatus. Using the cold-finger apparatus and pieces of the ganglion less than 0·5 mm thick, overnight drying at -40°C is satisfactory.

It is worthwhile for each investigator to find out the minimum drying time for his own apparatus. If overnight is sufficient, it is a tremendous waste of time to

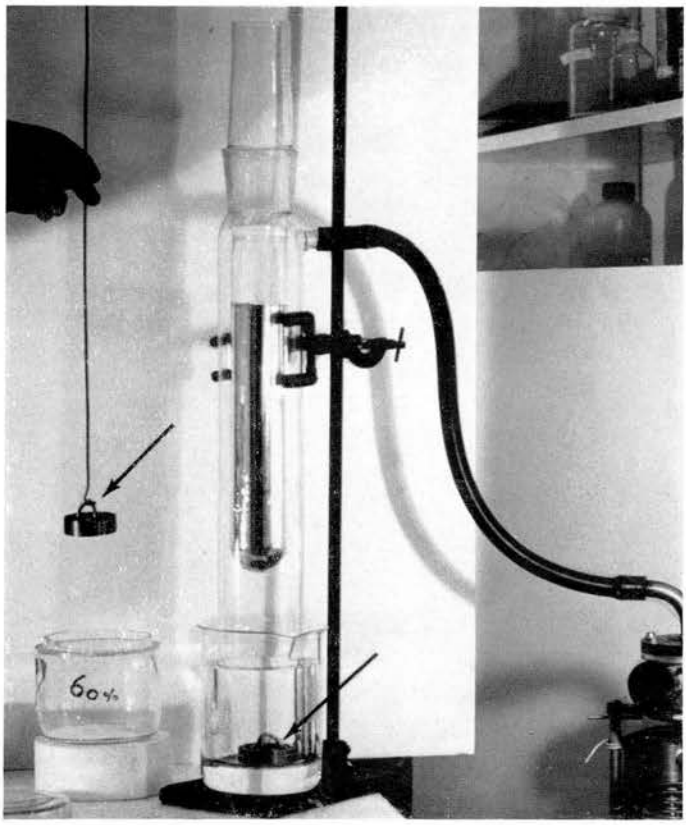


Fig. 4. The apparatus shown in fig. 3 immersed in warm water at the end of the drying process, so as to warm up the tissues in the copper tissue holder (arrow) before opening the apparatus. On the left another holder (arrow) on the way to the paraformaldehyde vessel.

dry for a week. On the other hand, specimens dried too hastily may be misleading in preserving some of the amines, say in the mast cells, but allowing the less firmly bound ones, *e.g.* catecholamines in the nerve fibres, to diffuse during the warming-up period.

Lowering the temperature prolongs the drying time, and most workers seem to dry at about -40° for FIF. Relatively little high magnification work has as yet been carried out, and it is thus somewhat open to doubt whether better localization would be achieved by drying at a lower temperature. Some observations (unpublished) with 1 μ sections of material embedded in epoxy resin suggest that -50° might be better.

Before removing the tissue from the freeze-drying apparatus, the temperature of the pieces must be raised well above the ambient, otherwise atmospheric water vapour will condense on the specimen and ruin it. Freeze-dried tissue pieces, even at the ambient temperature, are quite hygroscopic and, unless the relative humidity of the air is 30 p.c. or less (one of the few advantages of a cold climate), the tissue must be handled in chambers kept dry with a desiccant such as silica gel.

It is safest to warm up the tissue holder to about 50°C, raising the temperature gradually and allowing for the considerable heat-absorbing capacity of the metal. The procedure employed in the author's laboratory is to take the apparatus out of the deep freeze for 2 hours and then to stand it for 1 hour in water as warm as the fingers can stand, before breaking the vacuum (fig. 4). The tissue holder is then transferred with the tissue pieces into glass containers (shown on the left in fig. 4) for exposure to formaldehyde vapour.

(3) *Exposure to formaldehyde*

The freeze-dried tissue pieces are exposed to formaldehyde vapour, which is generated by warming paraformaldehyde powder in a glass container. In the first studies, capricious results were often obtained. The method became much easier, when Hamberger *et al.*, (1965) pointed out the importance of the water content of the paraformaldehyde powder and proposed its standardization in a desiccator over a solution of sulphuric acid. A series of batches providing a relative humidity of 50–80 p.c. was recommended. With such a series a suitable humidity must be determined empirically for each tissue (fig. 5).

A temperature of 80°C is most commonly used (*e.g.*, Falck & Owman, 1965). However, a lower temperature is often preferable; demonstration of many enzymes is indeed possible in a section in which the FIF has first been registered at a moderate temperature (Eränkö, 1964). However, when the temperature is lowered, the water content of the paraformaldehyde powder must be increased. If the exposure time is about one hour, satisfactory results can be obtained by adjusting the water content of paraformaldehyde and the temperature according to a "rule of 110". This means that the sum of the percentage relative humidity of air with which the paraformaldehyde powder has been equilibriated and the temperature of exposure (in degrees Centigrade) must be 110. Table 1 shows the relative humidity of air at 20°C over solutions of sulphuric acid of different concentrations and specific gravities.

As was pointed out by Hamberger *et al.* (1965), different tissues require different exposure conditions for the development of an optimum FIF. As an example an observation by Fuxe (1965) may be mentioned; demonstration of the nerve terminals containing 5-hydroxytryptamine in the central nervous system require much more humid exposure conditions than the catecholamine-containing terminals in the same specimen so that diffusion is often manifest in the latter. However, discussions

Fig. 5. A series of 1 lb. glass cylinders for formaldehyde gassing. In the centre, a petri dish with a watch glass and a humido-thermometer are shown. Note the extremely low atmospheric relative humidity, prevalent most of the time in Finland.

Table 1. Relative humidity of air at 20°C above different concentrations of sulphuric acid (calculated from data provided in Hodgman (1949)).

Relative humidity (p.c.)	P.c. of H_2SO_4	Density of H_2SO_4
20	60	1·50
30	54	1·44
40	49	1·39
50	43	1·33
60	41	1·31
70	34	1·25
80	28	1·20
90	20	1·14

with a dozen workers employing, after independent trial and error, very different conditions have indicated that the rule of 110 is a good approximation.

In practice, 4 desiccators providing relative humidities of 50, 60, 70, and 80 p.c. are enough. A sufficient range of exposure conditions for all tissues can then be obtained by varying the temperature from 30 to 90°C. In the first trial, a temperature of 50°C is recommended together with paraformaldehyde powder equilibriated at 60 p.c. humidity.

For exposure, the freeze-dried tissue piece can be placed on a watch glass, which is then placed in a 5″ diameter Petri dish, on the bottom of which is spread the paraformaldehyde powder fresh from the desiccator (fig. 5). The dish is then transferred to an incubating oven, which must be kept in a well ventilated place such as a fume cupboard. Alternatively, the metal tissue holder is transferred while still warm to a larger glass vessel (fig. 4) which contains paraformaldehyde powder on the bottom. The amount of powder is not very critical. While Falck and Owman (1965) use 6 gm for a 1 litre vessel, in our laboratory a teaspoon of powder is spread in a 1 lb. jam jar.

(4) *Embedding, sectioning, and mounting*

After exposure to formaldehyde vapour, the tissue must be embedded for sectioning. Paraffin wax is the most commonly used medium, and vacuum embedding is the quickest way to impregnate the tissue. A vacuum oven is convenient for this purpose but by no means essential. The paraffin wax must be previously melted and degassed by evacuation with a mechanical pump until bubble formation ceases (usually about 15 min.). For vacuum embeddings, the tissue is evacuated in the same holder with molten paraffin wax for 5 min. The wax is then poured on the tissue pieces by tilting the holder, and the vacuum is broken.

The pieces can also be embedded more conventionally by transferring them first into xylene and then into four changes of wax, 5–10 min. each. However, this method may not be good for frog tissues, in which adrenaline is the predominant amine, because the fluorochrome from adrenaline has been claimed to dissolve in xylene (Falck & Owman, 1965).

Instead of paraffin wax, which does not allow the cutting of sections much thinner than 5 μ, epoxy resins can be advantageously used as embedding media in the same manner as in electron microscopy (Hökfelt, 1965). This makes it possible to cut very thin sections, which naturally improves the resolution; 1 μ resin sections show much sharper details than 5 μ paraffin sections.

Xylene, liquid paraffin and Entellan (E. Merck) are suitable mounting media, while the intense fluorescence of Canada balsam is prohibitive. For stretching the sections, water cannot be employed because the fluorescent compounds then become displaced. When cutting paraffin sections, care should be taken to obtain flat ones; it is then sufficient to allow them to melt on a warm slide. The molten section is covered with a small drop of the mounting medium, and the cover slip is placed over the drop. Smith (1966) has found butyl alcohol a convenient medium for stretching wrinkled paraffin sections, in the same way as water is ordinarily used; butyl alcohol can be expected to be serviceable also for epoxy-resin sections.

(5) *Fluorescence microscopy*

Despite a wide-spread misconception, complicated apparatus or special lenses are not necessary for fluorescence microscopy. Almost any microscope is good enough for the purpose, provided its optical components are non-fluorescent. Quartz objectives and oculars offer no advantage over glass ones, provided only visible fluorescence is being studied. A quartz condenser is likewise unnecessary for most work; any advantage it may have over a glass condenser in transmitting ultraviolet light of lower wavelengths is lost, unless quartz slides are used as well. For qualitative catecholamine work, glass lenses throughout are perfectly satisfactory.

A dark-ground condenser of the cardioid type is to be recommended, not only because it makes possible the use of efficient filter combinations which transmit some visible exciting light, but also because it provides a clean background by minimizing the fluorescence of the microscope objective and of the secondary filter, which often exhibits a marked fluorescence. The use of an immersion fluid between the condenser and the slide is to be recommended, even if a bright-field condenser is used, because the illumination intensity increases as the square of the condenser aperture, and the fluid increases the effective numerical aperture from 0·9 to 1·4. This more than doubles the intensity of the fluorescence. Ordinary non-fluorescent glycerol can be used as the immersion medium; it is inexpensive, easy to clean and does not smell.

A monocular microscope with the minimum of prisms and mirrors in the optical path is the best. Figure 6 shows a simple photomicrography set-up used in the author's laboratory. During visual inspection, all the fluorescent light is deviated

to the side tube by a prism but during photographic exposure the prism is displaced and all the light falls on the photographic emulsion; thus maximum efficiency is obtained. This is desirable because the FIF due to all amines fades in ultraviolet and blue light.

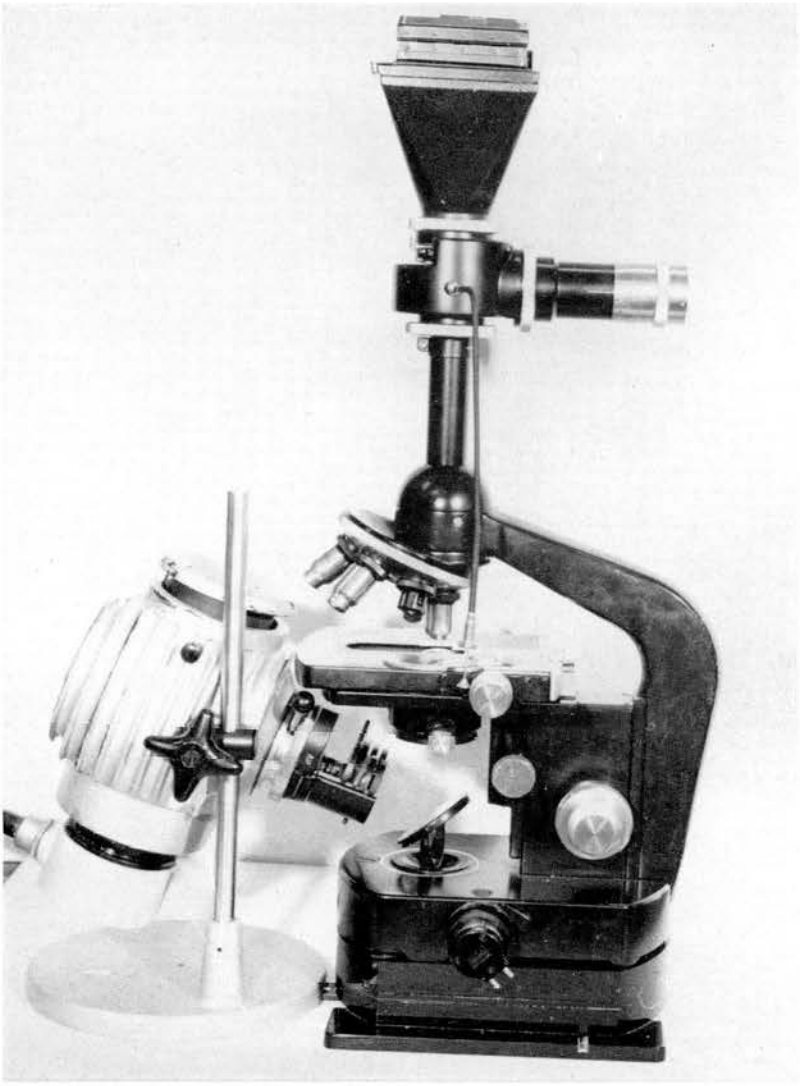


Fig. 6. A simple but efficient fluorescence photomicrography arrangement. All the fluorescent light is falling on the emulsion during exposure, the prism being then deviated from the optical path.

For this reason, it is also preferable to use high-aperture objectives such as apochromats or planachromats and the smallest ocular magnification possible, since the illumination intensity in the image plane is proportional to the square of the objective aperture but inversely proportional to the square of the ocular magnification. A short distance from the ocular to the photographic emulsion serves the same light-saving purpose.

The microscope lamp must provide ultraviolet and blue light of sufficient intensity. It must also be close enough to the condenser to illuminate a reasonably large field in the object plane when a high-aperture immersion condenser is used (*cf.* fig. 6). The former condition is at present met in most commercially available lamps, which use the high pressure mercury lamp Osram HBO 200. The lamp condensers are also generally satisfactory, but it may be an advantage to acquire one made of quartz.

Three types of filters are necessary: (1) heat absorbing filters, such as the Schott KG 1 and BG 38, of which the latter also cuts out the red end of the spectrum passed by many ultraviolet filters; (2) ultraviolet or blue filters for producing the exciting light; and (3) yellow or orange filters extinguishing the exciting light but passing the fluorescence. The heat-absorbing and ultraviolet exciter filters are conveniently placed directly in front of the lamp condenser; the yellow secondary filter should be placed between the objective and the ocular. Unfortunately, not all microscopes have adequate filter holders in this site to provide for rapid changing of the secondary filters.

The best all-round filter combination for FIF consists of the Schott BG 12 as a blue filter and the OG 1 (preferably combined with a GG 11 to diminish the intense filter fluorescence of OG 1) as a secondary filter. The transmission spectra of these filters are shown in fig. 7.

Another commonly used pair (also illustrated in fig. 7) consists of filters UG 1 and GG 13 comb.; this is better in passing fluorescent light of shorter wavelengths. It can be recommended especially when it is desirable to study the fluorescence colour. However, the ultraviolet light passed by UG 1 is not very efficient in exciting the FIF of monoamines, which makes the over-all intensity weak. The filter GG 13 comb. is a development of the filter GG 13, and cuts off rather more of the light transmitted by UG 1. Filter GG 13 comb. is thus more efficient than filter GG 14, when used in combination with UG 1. The spectra of these secondary filters are shown in fig. 8, together with the emission spectra of the FIF of noradrenaline and 5-hydroxytryptamine.

Fluorescence photomicrographs representing different types of tissues and different photographic conditions are illustrated in the Colour Plate. Figure 9 shows a section of freeze-dried adrenal medulla exposed to formaldehyde vapour of 50 p.c. humidity equilibration for 15 min. at 50°C. Because of the mild exposure, a strong fluorescence is present only in the typical noradrenaline cell islets, while the adrenaline-containing cells fluoresce but weakly. After exposure for 2 hours at 80°C, all cells fluoresce with equal intensity. The yellow colour of the noradrenaline cell islets is a typical example of the colour-shifting effect of an increase in the fluorescence intensity; the emission spectrum of these yellow islets and that of the green background are actually similar.

Figure 10 shows a typical rat iris with intensely fluorescent nerve fibres exhibiting synaptic varicosities. Both the circular sphincter and the radial dilator muscle contain such fibres. The preparation has been made by stretching a fresh iris on a slide, allowing it to dry, and exposing it subsequently to formaldehyde vapour. Fluorescent fibres similar to those in the rat iris are seen in the muscle of the guinea-pig ductus deferens in fig. 11.

The specimen shown in fig. 12 is from the para-aortic tissue of a newborn guinea pig. It is interesting in showing a ganglion composed of fluorescent nerve cells surrounded by numerous fluorescent nerve fibres, whose varicosities are apparently in synaptic contact with the cells. The nerve cells shown in the rat superior cervical ganglion (fig. 13) are more intensely fluorescent but the fluorescent fibres around them are fewer. These cells contain numerous granules in the cyto-

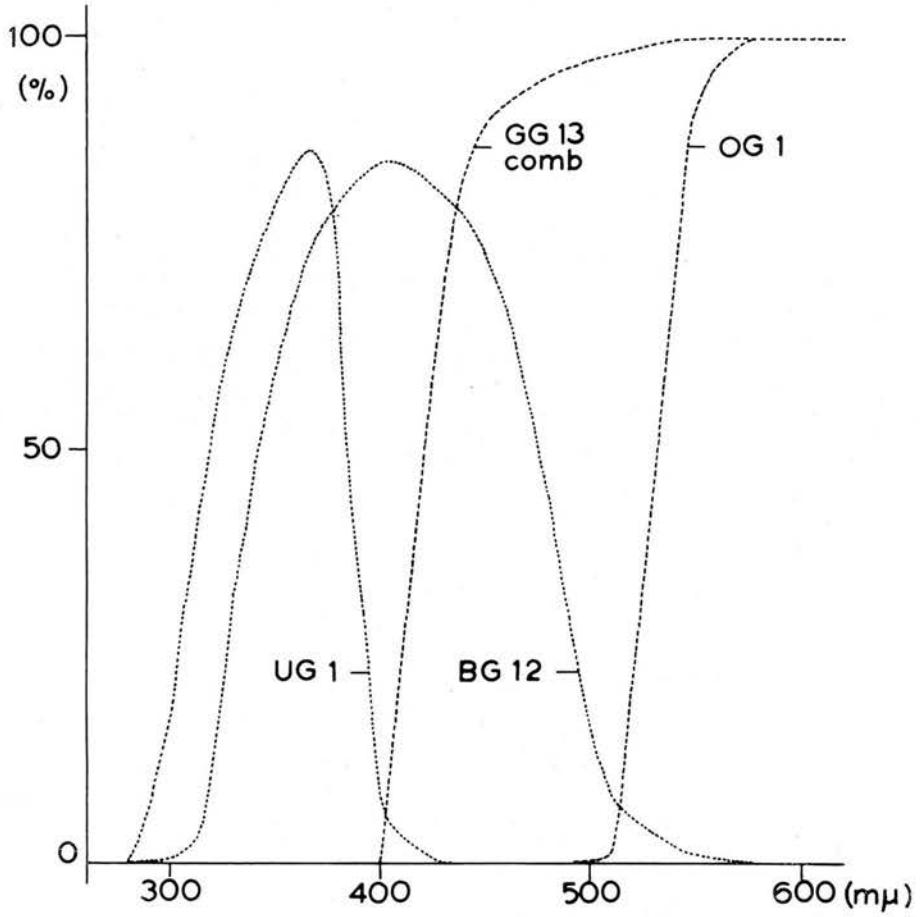


Fig. 7. Transmission spectra of some commonly used filters for fluorescence microscopy.

plasm exhibiting an orange-yellow fluorescence, which is not formaldehyde-induced but probably due to lysosomal lipids.

The strongly developed Auerbach plexus of the guinea-pig tænia coli is illustrated in fig. 14. It contains numerous intensely fluorescent adrenergic synapses in contact with the cholinergic nerve-cell bodies and nerve fibres of the plexus. Figure 15, taken tangentially from the circular muscle of the cat duodenum, illustrates the mode of adrenergic innervation typical of this muscle.

Figures 16–18 show the same preparation of rat connective tissue with typical noradrenaline-containing nerve fibres and two 5-hydroxytryptamine-containing mast cells, photographed using different filter combinations. Best colour differentiation between the nerve fibres and the mast cells is obtained in fig. 18. Note that some of the blue light is transmitted in spite of the dark-ground illumination.

Normal photographic materials are suitable for fluorescence photomicrography. It is often better to employ a 35 mm film of medium speed (24–27 Din or 200–400 ASA) than a 120 size maximum speed film. Maximum speed emulsions have a disproportionately large grain size and often they are not really much more sensitive for fluorescence photography, in which reciprocity failure due to low light

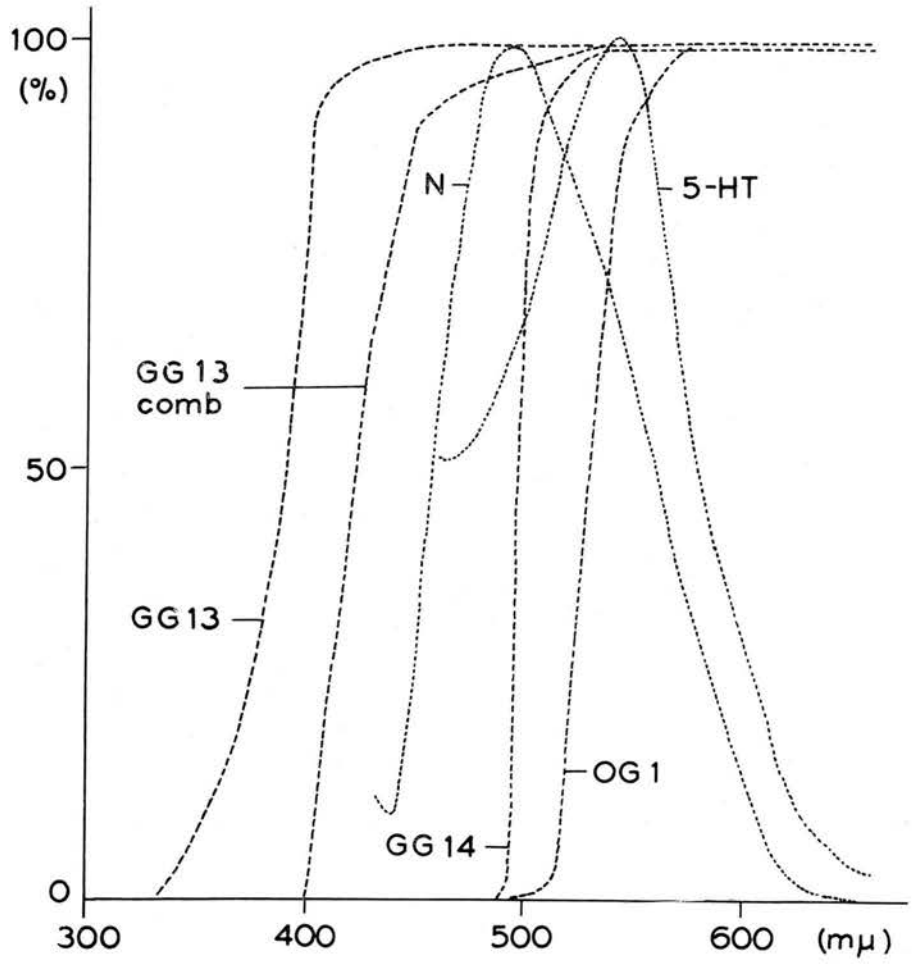


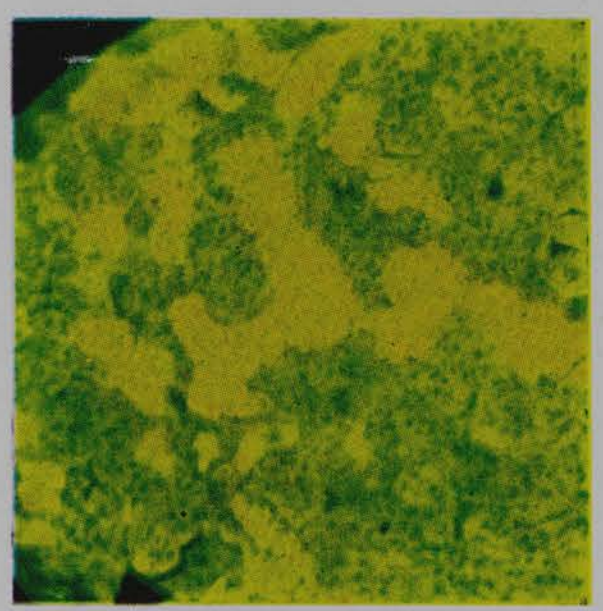
Fig. 8. Transmission spectra of some secondary filters and the emission spectra of the fluorescence produced by formaldehyde from noradrenaline (N) and 5-hydroxytryptamine (5-HT).

levels complicates the matter. Satisfactory colour photographs can be obtained using either daylight or artificial light films, but the former are recommended because of their higher relative sensitivity to yellow and red light, which is important in fluorescence work.

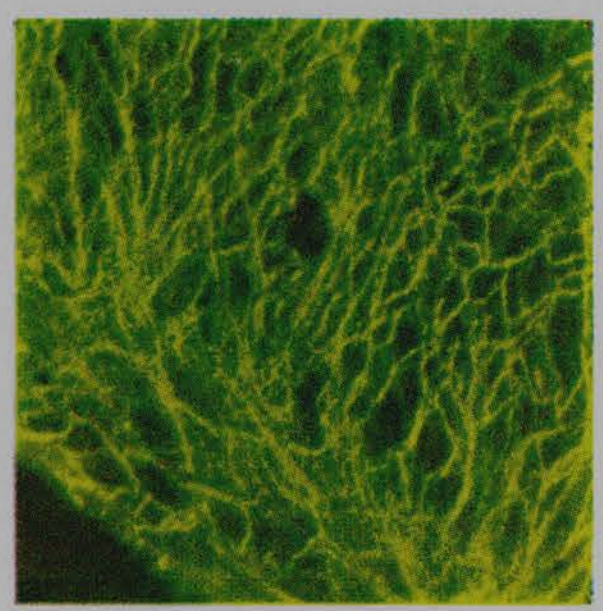
The author has carried out some experiments (unpublished) with the false-colour Aero Ektachrome infrared film. This differentiates beautifully, with bright colours, between various brownish objects in aerial photographs (Tarkington & Sorem, 1963). In fluorescence microscopy, the results were disappointing but the principle of colour translation should be applicable, if it should prove possible to apply it to the region 500–700 mμ of the visible spectrum.

(6) *Discrimination between different amines*

Differences in the rate of formation, in the spectral characteristics and the reactions of the fluorescent compounds formed from different amines can be used to discriminate between them.

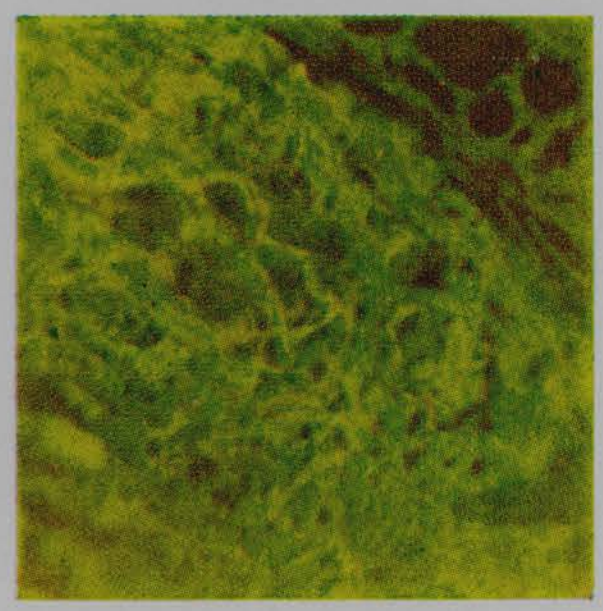


9

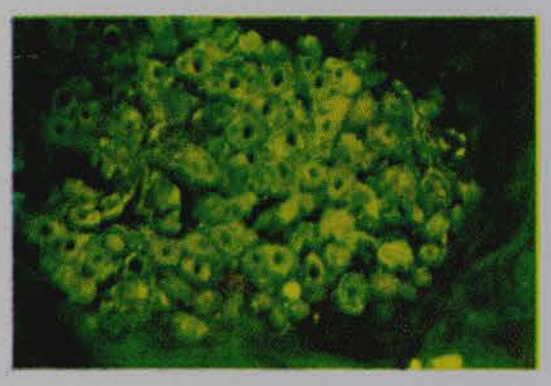


10

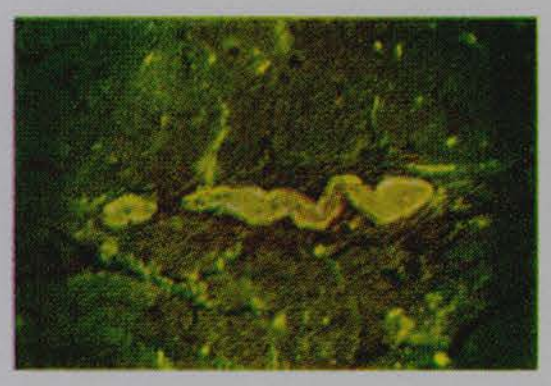
11



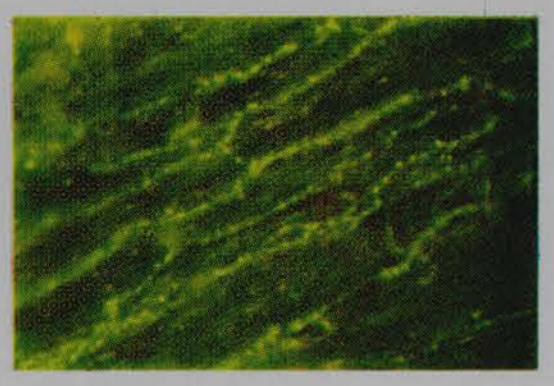
12



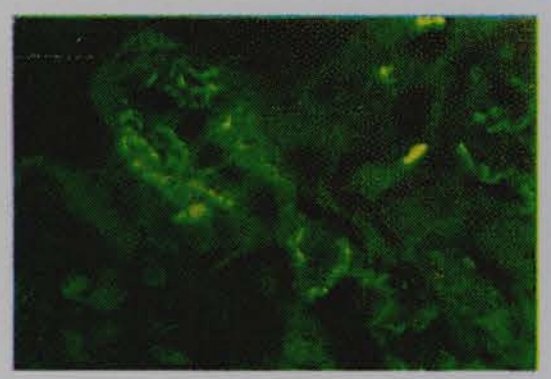
13



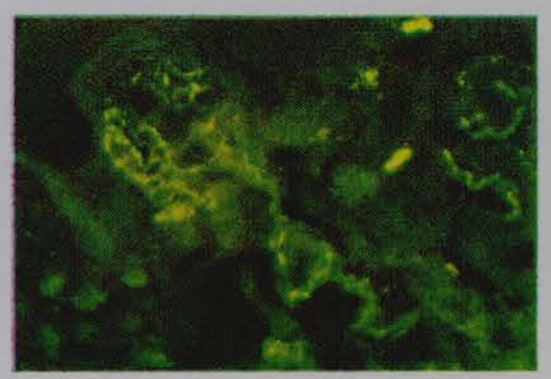
14



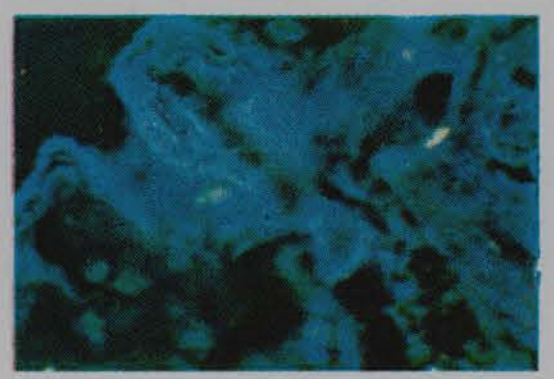
15



16



17



18

[*To face page 273.*

Figure 19 shows the excitation and emission spectra of the fluorescent compounds formed from noradrenaline and 5-hydroxytryptamine, respectively (Corrodi & Jonsson, 1965*a*, *b*). Differences are obvious both in the excitation spectra and in the emission spectra. Emission differences are more or less clearly apparent in ordinary fluorescence microscopy as a difference in the fluorescence colour; the FIF reaction of noradrenaline-containing adrenergic nerve fibres is green, while that of 5-hydroxytryptamine in the mast cells is yellow. However, special care is necessary, when colour differences are used as guides to the nature of the amines demonstrated with the FIF method. Both the eye and the photographic colour emulsion tend to "see" green colour, such as that of the FIF due to noradrenaline, progressively more yellow when the intensity of the fluorescence is increased (fig. 9). Quantitative registration of the emission spectra is therefore highly desirable, and a relatively simple apparatus for microscopic spectrophotofluorometry has been described recently by Thieme (1966). For further details of this method the reader is referred to the recent paper by Caspersson *et al.* (1966).

Because the emission spectra of dopamine, noradrenaline and adrenaline are essentially similar, other means must be employed to discriminate between them. Adrenaline differs from dopamine and noradrenaline in being, as a secondary

Fig. 9. Freeze-dried rat's adrenal medulla exposed for 15 min. at 50°C to formaldehyde vapour equilibriated with 50 p.c. humidity. Noradrenaline cell islets exhibit bright yellow fluorescence, while the adrenaline cells fluoresce with a less intense green colour. Dark ground, BG 12, OG 1. ×75.

Fig. 10. Stretch preparation of a rat iris exposed after drying for 30 min. to formaldehyde vapour at 50°C equilibriated with 60 p.c. humidity. Both the sphincter and the dilator muscle show numerous adrenergic fibres with synaptic varicosities. Dark ground, BG 12, OG 1. ×150.

Fig. 11. FIF in the cross section of the ductus deferens of the guinea-pig. An extremely dense network of adrenergic fibres is visible in the smooth muscle, while the mucosa is non-fluorescent. Dark ground, BG 12, OG 1. ×75.

Fig. 12. Sympathetic ganglion in the para-aortic region of a newborn guinea-pig. Fluorescent ganglion cells are surrounded by numerous fluorescent fibres with synaptic varicosities. Bright field, BG 12, OG 1. ×300.

Fig. 13. Superior cervical ganglion of the rat. Orange autofluorescent granules are seen in the cytoplasm of the nerve cells with an intense FIF. Dark ground, BG 12, GG 14. ×150.

Fig. 14. Guinea-pig colon showing Auerbach's plexus with intensely fluorescent adrenergic synapses. Some adrenergic fibres are also visible on both sides of the plexus. Dark ground, BG 3, GG 14. ×65.

Fig. 15. Adrenergic nerve net in the smooth muscle of the cat duodenum. The synaptic varicosities are intensely fluorescent, while other parts of nerve fibres are poorly visible. Orange granules are due to autofluorescence. Dark ground, BG 3, GG 14. ×125.

Fig. 16. Adrenergic nerve fibres in loose connective tissue of the rat. One thick and another thin mast cell are also visible; their FIF is due to 5-hydroxytryptamine. All structures fluoresce with a green colour. Dark ground, BG 3, GG 11, and VG 4. ×125.

Fig. 17. The same preparation photographed with different filters. Mast cells are yellowish, nerve fibres are green, but the colour difference is not very striking. Dark ground, BG 12, OG 1. ×125.

Fig. 18. The same preparation, again with a different filter combination. The secondary filter VG 9 passes some of the ultraviolet light filtered through UG 1 but, because of dark ground illumination, this does not prevent examination. Distinct colour difference is evident between nerve fibres and mast cells. Dark ground, UG 1, VG 9. ×125.

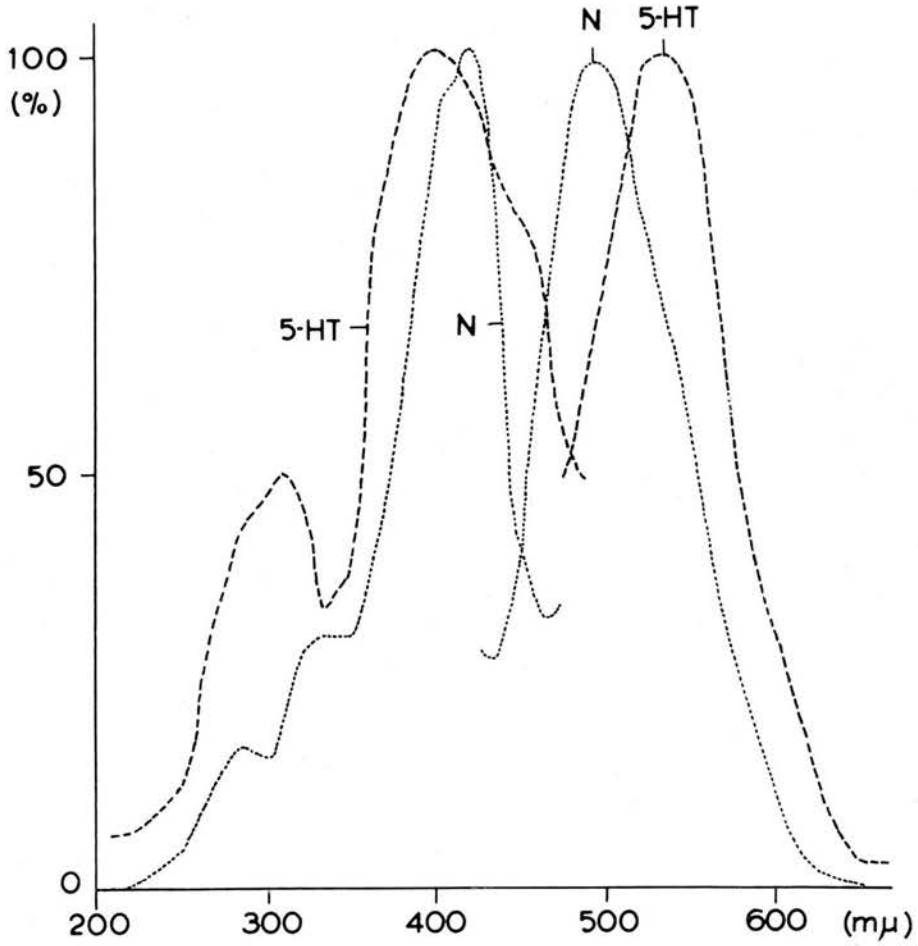


Fig. 19. Excitation (left) and emission (right) spectra of the fluorescent products from noradrenaline (N) and 5-hydroxytryptamine (5-HT). Note the marked overlapping in the emission spectra, in spite of the clearly separated emission maxima.

amine, more slowly condensed with formaldehyde (Eränkö, 1964; Falck, 1962). Therefore, fluorescence appearing first after a prolonged exposure to formaldehyde can be expected to be due to adrenaline.

Differentiation between dopamine and noradrenaline can be effected using thionyl chloride. This splits off the hydroxy-group at the position 4 in the 3,4-dihydroisoquinoline derivative of noradrenaline, forming a fully aromatic, 6,7-dihydroxyisoquinoline (Corrodi & Jonsson, 1965*a*). This compound retains its fluorescence after treatment with sodium borohydride, while that obtained in a similar way from dopamine is reduced and becomes non-fluorescent.

References

Acheson, G. H. (Editor) (1965)—"Second symposium on catecholamines". *Pharmacol. Revs.*, **18**, 1.

Barter, R. & Pearse, A. G. E. (1953).—Detection of 5-hydroxytryptamine in mammalian enterochromaffin cells. *Nature (Lond.)*, **172**, 810.

Barter, R. & Pearse, A.G.E. (1955).—Mammalian enterochromaffin cells as the source of serotonin (5-hydroxytryptamine). *J. Path. Bact.*, **69**, 25.

Carlsson, A., Falck, B., & Hillarp, N.-Å. (1962).—Cellular localization of brain monoamines. *Acta Physiol. scand.*, **56**, Supp. 196.

Caspersson, T., Hillarp, N.-Å., & Ritzen, M. (1966).—Fluorescence microspectrophotometry of cellular catecholamines and 5-hydroxytryptamine. *Exptl. Cell. Res.*, **42**, 415.

Corrodi, H. & Hillarp, N.-Å. (1963).—Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6,7-Dimethoxyisochinolinderivaten und Formaldehyd. *Helv. Chim. Acta*, **46**, 2425.

—— & —— (1964).—Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. *Helv. Chim. Acta.*, **47**, 911.

——, ——, & Jonsson, G. (1964).—Fluorescence methods for the histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. *J. Histochem. Cytochem.*, **12**, 582.

—— & Jonsson, G. (1965*a*).—Fluorescence methods for the histochemical demonstration of monoamines. 4. Histochemical differentiation between dopamine and noradrenaline in models. *J. Histochem. Cytochem.*, **13**, 484.

—— & —— (1965*b*).—Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 5. Identifizierung des fluoreszierenden Produktes aus Modellversuchen mit 5-Methoxytryptamin und Formaldehyd. *Acta histochem.*, **22**, 247.

Dahlström, A. & Fuxe, K. (1964).—Evidence for the existence of monoamine neurons in the central nervous system. I. *Acta physiol. scand.*, **62**, Supp. 232.

—— & —— (1965).—Evidence for the existence of monoamine neurons in the central nervous system. II. *Acta physiol. scand.*, **64**, Supp. 247.

Eränkö, O. (1951).—On the histochemistry of the rat adrenal medulla. *Acta physiol. scand.*, **25**, Supp. 89.

—— (1952).—On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. *Acta anat.*, **16**, Supp. 17.

—— (1954*a*).—Quenching of tissues for freeze-drying. *Acta anat.*, **22**, 331.

—— (1954*b*).—A simple apparatus for freeze-drying of animal tissues. *Acta path. microbiol. scand.*, **35**, 426.

—— (1955).—Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. *Acta endocrinol.*, **18**, 174.

—— (1958).—"Studies on the adrenal medulla". Thesis for the Degree of Ph. D., University of Edinburgh.

—— (1961).—Personal communication acknowledged in Falck and Torp (1961).

—— (1964).—Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.*, **12**, 487.

—— (1966).—Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *Proc. roy. micr. Soc.*, **1**, 209.

—— (1967).—Histochemistry of nervous tissues: catecholamines and cholinesterases. *Ann. Rev. Pharmacol.*, **7**, 203.

—— & Kallio, H. (1967).—Apparatus for rapid freezing of tissues. *J. Histochem. Cytochem.*, in press.

—— & Räisänen, L. (1966).—Demonstration of catecholamines in adrenergic nerve fibres by fixation in aqueous formaldehyde solution and fluorescence microscopy. *J. Histochem. Cytochem.*, **14**, 690.

Erös, G. (1932).—Eine neue Darstellungsmethode der sogenannten "gelben" argentaffinen Zellen des Magendarmtraktes. *Centralbl. allg. Path. path. Anat.*, **54**, 385.

Euler, U. S. von, Rosell, S. & Uvnäs, B. (Editors) (1965).—"Mechanisms of release of biogenic amines". Pergamon Press, Oxford.

Falck, B. (1962).—Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.*, **56**, Supp. 197.

——, Hillarp, N.-Å., Thieme, G., & Torp, A. (1962).—Fluorescence of catechol amines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.*, **10**, 348.

—— & Owman, C. (1965).—A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta. Univ. Lund. Sect. II*, No. 7.

—— & Torp, A. (1961).—A fluorescence method for histochemical demonstration of noradrenalin in the adrenal medulla. *Med. Exptl. (Basle)*, **5**, 429.

Fuxe, K. (1965).—"Evidence for the existence of monoamine neurons in the central nervous system". Almqvist & Wiksells, Uppsala.

Hamberger, B., Malmfors, T., & Sachs, C. (1965).—Standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines. *J. Histochem. Cytochem.*, **13**, 147.

Harreveld, A. van & Crowell, J. (1964).—Electron microscopy after rapid freezing on a metal surface and substitution fixation. *Anat. Rec.*, **149**, 381.

Hodgman, C. D. (Editor) (1949).—"Handbook of chemistry and physics", 21st ed. Chemical Rubber Publishing Co. Cleveland, Ohio.

Hökfelt, T. (1965).—A modification of the histochemical fluorescence method for the demonstration of catecholamines and 5-hydroxytryptamine, using araldite as embedding medium. *J. Histochem. Cytochem.*, **13**, 518.

Jacobowitz, D. & Koelle, G. B. (1965).—Histochemical correlations of acetylcholinesterase and catecholamines in postganglionic autonomic nerves of the cat, rabbit, and guinea pig. *J. Pharm. exp. Ther.*, **148**, 225.

Kokko, A. (1965).—Histochemical and cytophotometric observations on esterases in the spinal ganglion of the rat. *Acta physiol. scand.*, **66**, 261.

Lagunoff, D., Phillips, M., & Benditt, E. V. (1961).—The histochemical demonstration of histamine in mast cells. *J. Histochem. Cytochem.*, **9**, 534.

Lempinen, M. (1966).—Personal communication.

Majno, G. & Bunker, W. E. (1957).—Preparation of tissue slices for metabolic studies: a hand-microtome especially suitable for brain. *J. Neurochem.*, **2**, 11.

Malmfors, T. (1965).—Studies on adrenergic nerves. *Acta physiol. scand.*, **64**, Supp. 248.

Norberg, K.-A. & Hamberger, B. (1964).—The sympathetic adrenergic neuron. Some characteristics revealed by histochemical studies on the intraneuronal distribution of the transmitter. *Acta physiol. scand.*, **63**, Supp. 238.

Pearse, A. G. E. (1960).—"Histochemistry, theoretical and applied". Churchill, London.

—— (1953).—Rapid freeze-drying of biological tissues with a thermoelectric unit. *J. Sci. Instr.*, **40**, 176.

Smith, G. (1966).—Personal communication.

Tarkington, R. G. & Sorem, A. L. (1963).—Color and false-color films for aerial photography. *Photogrammetric Engineering*, **29**, 88.

Thieme, G. (1965).—Small tissue dryers with high capacity for rapid freeze-drying. *J. Histochem. Cytochem.*, **13**, 386.

—— (1966).—A versatile device for microscopic spectrofluorometry. *Acta physiol. scand.*, **67**, 514.

Printed in Great Britain by
William Clowes and Sons Ltd, London and Beccles

THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 15, No. 7

Printed in U.S.A.

DISTRIBUTION OF ESTERASES IN THE MYONEURAL JUNCTION OF THE STRIATED MUSCLE OF THE RAT

O. ERÄNKÖ AND H. TERÄVÄINEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received for publication December 7, 1966

Distribution of esterases in the myoneural junction of the striated muscle of the rat was studied using acetylthiocholine, butyrylthiocholine and α-naphthyl acetate as substrates, together with selective inhibitors.

Acetylcholinesterase activity was observed in the peripheral complex of synaptic folds. Nonspecific cholinesterase was detected in the peripheral complex of synaptic folds and the teloglia with approximately equal activities. Nonspecific esterase activity, present in tissues incubated with eserine, was marked in the terminal axon and was also present in the teloglia and synaptic folds.

Since 1949 (22) the myoneural junction has been shown by many investigators to be rich in cholinesterase activity. A number of workers have used acetylthiocholine as a substrate without inhibitors (26, 28, 29, 31, 33); they were therefore unable to discriminate between different cholinesterases, as were those using thiocholine with eserine (3, 4, 18) which inhibits equally both types of cholinesterases. Those authors who reported the use of even less specific substrates, such as α-naphthyl acetate, failed, moreover, to discriminate between nonspecific esterases and cholinesterases (1, 2, 15, 25, 34).

When correctly used with thiocholine substrates, diisopropyl fluorophosphonate (DFP) is adequate for discriminating between acetylcholinesterase (AChE) and nonspecific cholinesterase (nsChE) (12, 13, 30), but, because of limited selectivity, this inhibitor may also be insufficient (10, 14, 19). When more selective inhibitors, such as tetraisopropylpyrophosphoramide (iso-OMPA), 1,5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide(62C47) and 1,5-bis-(4-allyl dimethylammoniumphenyl) pentan-3-one diiodide (284C51), have been used (8, 9, 16, 20, 23, 27, 32) evidence has usually been obtained of the presence of both AChE and nonspecific cholinesterase in the end plate.

However, there is still some controversy concerning the localization of cholinesterases in the end plate. For example, AChE activity has been reported by some workers as confined exclusively in membranes of the terminal axon and subneural apparatus, in addition to the material filling the synaptic clefts (3, 25, 26), while other authors have observed a positive reaction also in the terminal sarcoplasm (29), in sarcoplasmic mitochondria (34), in synaptic vesicles of the terminal axon (1, 29) and in the teloglial cells (8, 27, 34). On the other hand, the presence of nonspecific esterases has, as a rule, been overlooked, and any esterase reaction seen in the motor end plate has often been interpreted as due to cholinesterase activity (1, 2, 15, 25, 34). Re-examination of the motor end plate, using techniques which would discriminate between AChE, nonspecific ChE and nonspecific esterases, was thus motivated.

We are aware of the oversimplification of a division of esterase activity into these three categories, and the reader who is interested in the problem of the multiplicity of esterases is referred to previous publications from our laboratory (11, 17, 24); in these the problem has been approached by using many substrates and inhibitors with both fresh and fixed tissue sections and zymograms, and at the same time the literature has also been reviewed. The schematic division of three predominant types of carboxylic esterase activity serves a useful purpose. On the basis of previous experience with other tissues (11, 17, 24), we believe that there is little overlapping between reactions obtained with the three substrate-inhibitor combinations employed in the present study.

MATERIAL AND METHODS

Albino rats of both sexes were killed under ether anesthesia by severing the neck. The tibialis anterior and gastrocnemius muscles and the dia-

phragm were dissected out. One portion of the muscles was frozen in isopentane cooled with liquid nitrogen and then cut in a cryostat. The sections were allowed to thaw and dry on coverslips. Other parts of the muscles were fixed fresh at about 4°C in a solution containing 1 volume of 35% HCHO, 6 volumes of 2% $CaCl_2$ and 3 volumes of H_2O. Fixation was continued from 4–144 hr. The pieces were washed in distilled water for 30 min before incubation. Fresh and fixed tissues showed the same distribution of cholinesterase activities. Only E-600-sensitive nonspecific esterase activity was demonstrable in unfixed tissues, while nonspecific esterase activity resistant to E-600 was seen only in fixed preparations (11, 17, 24).

For the demonstration of AChE activity, acetylthiocholine iodide was used as substrate with 10^{-6}–10^{-4} *M* iso-OMPA to inhibit nonspecific cholinesterase. Nonspecific cholinesterase activity was demonstrated using butyrylthiocholine iodide as substrate and 10^{-5}–10^{-3} *M* 284C51 to inhibit AChE. The method used was based on Gomori's modification (14) of the Koelle technique (21) Incubation times at 37°C were: 30 min–1 hr for AChE and approximately 3 hr for nonspecific ChE. Preincubation for 30 min in a substrate-free mixture containing the same concentration of inhibitor as the incubation solution was always carried out. Control sections, incubated with both 10^{-5} *M* iso-OMPA and 10^{-4} *M* 284C51, were negative.

Nonspecific esterase activities were demonstrated by using α-naphthyl acetate as substrate and fast blue RR salt as coupling agent. To inhibit cholinesterases, 10^{-5}–10^{-2} *M* eserine was employed. To discriminate between organophosphorus-resistant and -sensitive nonspecific esterases hydrolyzing α-naphthyl acetate, 10^{-5}–10^{-3} *M* diethyl *p*-nitrophenyl phosphate (E-600) was used. Incubation with eserine was carried out at room temperature for 6–17 min and with E-600 for 30–45 min by continuously filtering fresh mixture on the sections.

RESULTS

Acetylcholinesterase: The AChE reaction was observed mainly in the peripheral complex of synaptic folds (F); it was weaker in the center where the terminal axon (A) is located (Figs. 1 and 2). There was no staining in the teloglia or muscle.

Nonspecific cholinesterases: At low magnification, the distribution of nonspecific ChE activity appeared to be the same as that of AChE. Examination at a higher magnification, however, revealed differences (Fig. 3). The synaptic folds of the end plate reacted less intensely for nonspecific ChE than for AChE. Thus, nsChE activity of the fold complex was only slightly stronger than that of the central axonal part. A reaction was seen outside the end plate in what appeared to be teloglial cells (T) in the nonmyelinated part of the axon terminal (Figs. 3 and 4).

Nonspecific esterases: The distribution obtained without inhibitors with α-naphthyl acetate resembled that obtained with thiocholine substrates. The reaction was intense in the end plate after only 1–2 min of incubation. Because 10^{-5} *M* eserine had a strong inhibitory effect, most of this reaction was due to cholinesterases. After incubation from 6–9 min, however, a moderate reaction was observed also in the presence of eserine (Fig. 5). This activity, certainly not caused by cholinesterases since it was present even with 10^{-3} *M* eserine, was strongest in the central part occupied by the terminal axon (A); there was, however, a distinct, although weaker, reaction in the folds of the subneural apparatus (F) and in teloglial cells.

Since practically no reaction was obtained after incubation with α-naphthyl acetate for more than 10 min when 10^{-5} *M* E-600 was the inhibitor, the reaction obtained with eserine in 6–9 min was almost exclusively caused by the presence of E-600-sensitive nonspecific esterase, even in formalin-fixed tissue. Nevertheless, activity resistant to E-600 was observed in fixed tissues by incubation for approximately 30 min; the reaction so obtained

---

FIGS. 1–6. A, axon; F, fold complex; T, teloglial cells; N, motor nerve; MEP, motor end plate.

FIG. 1. AChE in a normal end plate. Note the intense reaction in the region of the subneural fold complex (F), as compared with the central region containing the axon (A). Acetylthiocholine as substrate and 10^{-5} *M* iso-OMPA. ×1950.

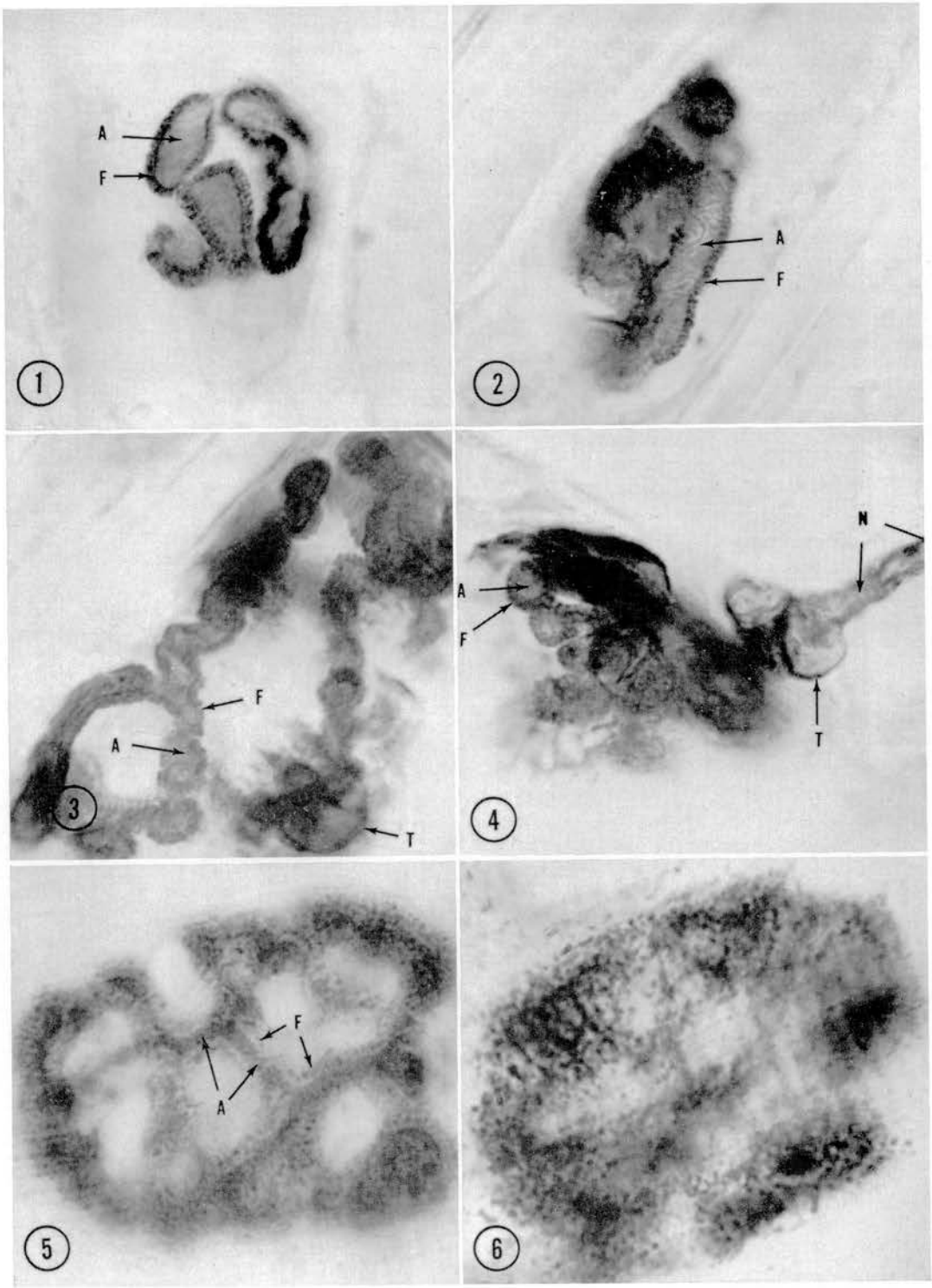
FIG. 2. AChE in a normal end plate, a part of which is out of focus. The synaptic folds are visible through the central part containing the axon. ×1950.

FIG. 3. nsChE in a normal end plate. Note staining in the teloglial cells (T). Butyrylthiocholine as substrate and 10^{-4} *M* 284C51. ×1950.

FIG. 4. nsChE in a side view of a normal end plate. Teloglial cells (T) are positive, and also a short distance along the nerve fiber (N). ×1950.

FIG. 5. nsE in a normal end plate. The neural part in the center (A) is more heavily stained than the lateral subneural part (F) of the terminal. Compare with Figure 1, in which the subneural part (F) is heavily stained for AChE. α-Naphthyl acetate as substrate and 10^{-5} *M* eserine. × 1950.

FIG. 6. Organophosphorus-resistant nsE in a normal end plate. α-Naphthyl acetate as substrate and 10^{-5} *M* E-600. × 2268.



FIGS. 1–6

was selectively located in the end plate and was, without doubt, due to an enzyme reaction. Its precise localization was difficult to place (Fig. 6). No staining was seen in control sections incubated for the same length of time without the substrate.

DISCUSSION

Our observations indicate that the rat myoneural junction exhibits both AChE and nonspecific ChE activity; this has already been reported previously (5, 8, 9, 16, 20, 23, 32). However, in the present work, a distinct difference was observed between the patterns of AChE and nsChE, and only the latter was present in the teloglial cells. Teloglial localization observed by investigators previously has been explained as the result of diffusion (5). However, an esterase activity in such a site has been observed also in electron microscopic studies "occasionally" (27, 34), "irregularly" (8) or "between the glial cells and the terminal axon" (25).

According to our observations, nonspecific cholinesterase activity is almost equally intense in the synaptic folds and in the center where the motor axon is situated, while AChE activity is principally found in the complex of the synaptic folds, as previously reported (5, 7). Couteaux (6) suggested in an early study that apparent presynaptic activity is merely due to an optical artifact. Presynaptic esterase activity has been reported in several electron microscopic studies (1, 3, 8, 25, 26, 27, 29, 34). However, in these studies nonspecific substrates such as α-naphthyl acetate or thiolacetic acid were employed without selective inhibitors which discriminate between AChE, nsChE and other carboxylic esterases.

Indeed, it is possible that nonspecific esterase activity has contributed to the presynaptic reaction observed in the earlier studies, in view of its preferential localization in the axon observed in the present study. Staining caused, beyond any doubt, by nonspecific esterase activity, since it occurred in the presence of 10^{-3} M eserine, was observed not only in the terminal axon but also in the subneural apparatus and the teloglia. The presence of nonspecific esterase activity in the motor end plate has been denied by Denz (9), but his use of unfixed, frozen sections may have been responsible for negative findings. Koelle and Gromadzki (23), with gold thiolacetate as the substrate, found no evidence of nonspecific esterase activity in the end plate, while Barrnett (1) has observed a positive reaction with thiolacetate in the presence of eserine. Tuncbay's observations with different substrates also suggest the presence of nsE, although she did not demonstrate eserine-resistant activity in the end plate (32).

While the present observations have not yielded information on the functional significance of nonspecific esterase in the motor end plate, they clearly demonstrate its presence there. They also emphasize the importance of using specific substrates and inhibitors in all esterase studies, rather than assuming, as has often been done, that any esterase reaction in the motor end plate is caused by AChE activity. Further studies are now in progress to assess the effect of nerve division upon the different types of esterase activity in the motor end plate.

REFERENCES

1. Barrnett, R. J.: The fine structural localization of acetylcholinesterase at the myoneural junction. *J. Cell Biol. 12:* 247, 1962.
2. Bauer, W. C., Blumberg, J. M. and Zacks, S. I.: Short and long term ultrastructure changes in denervated mouse motor end plates. In *Proceedings of the Fourth International Congress of Neuropathology, Munich, 1962*, edited by H. Jacob. Georg Thieme, Stuttgart, 1962, p. 16.
3. Birks, R. J. and Brown, L. M.: A method for locating cholinesterase of a mammalian myoneural junction by electron microscopy. *J. Physiol. (London) 152:* 5P, 1960.
4. Brzin, M. and Majcen-Tkacev, Z.: Cholinesterase in denervated end plates and muscle fibers. *J. Cell Biol. 19:* 349, 1963.
5. Couteaux, R.: Localization of cholinesterases at neuromuscular junctions. *Int. Rev. Cytol. 4:* 335, 1955.
6. Couteaux, R.: Morphological and cytochemical observations on the postsynaptic membrane of motor end plates and ganglionic synapses. *Exp. Cell Res.*, suppl. 5, 294, 1958.
7. Csillik, B.: Functional structure of the postsynaptic membrane in the myoneural junction. Akademiai Kiado, Budapest, 1965.
8. Dawis, R. and Koelle, G. B.: Electron microscopic localization of acetyl-cholinesterase at the motor end plates by the gold-thiolacetic acid and gold-thiocholine methods. *J. Histochem. Cytochem. 8:* 703, 1965.
9. Denz, F. A.: On the histochemistry of the myoneural junction. *Brit. J. Exp. Path. 34:* 329, 1953.
10. Eränkö, O.: Specific demonstration of acetylcholinesterase and non-specific cholinesterase in the adrenal gland of the rat. *Histochemie 1:* 257, 1959.
11. Eränkö, O., Härkönen, M., Kokko, A. and Räisänen, L.: Histochemical and starch-gel electrophoretic characterization of desmo- and lyoesterases in the sympathetic and spinal ganglia of the rat. *J. Histochem. Cytochem. 12:* 570, 1964.

12. Filogamo, G. and Gabella, G.: Cholinesterase in denervated and innervated muscles. *Acta Anat. 63:* 199, 1966.
13. Gerebtzoff, M. A., Philippot, E. and Dallemagne, M. J.: Recherches histochimiques sur les acetylcholine- et choline-esterase. *Acta Anat. 20:* 234, 1954.
14. Gomori, G.: *Microscopic Histochemistry. Principles and Practice.* The University of Chicago Press, Chicago, 1952.
15. Guth, L. and Zalewski, A.: Disposition of cholinesterases following implantation of nerve into innervated and denervated muscle. *Exp. Neurol. 7:* 316, 1963.
16. Häggqvist, G.: Cholinesterases and innervation of skeletal muscles. *Acta Physiol. Scand. 48:* 63, 1960.
17. Härkönen, M.: Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. *Acta Physiol. Scand. 63:* suppl. 237, 1, 1964.
18. Hines, M.: A histochemical study of the localization of cholinesterase in skeletal muscle during its regeneration. *Anat. Rec. 138:* 283, 1960.
19. Holmstedt, B. and Sjöqvist, F.: Some principles about histochemistry of cholinesterases with special reference to the thiocholine method. *Bibl. Anat. 2:* 1, 1961.
20. Koelle, G. B.: The histochemical differentiation of types of cholinesterase and their localization in tissues of the cat. *J. Pharmacol. Exp. Ther. 100:* 158, 1950.
21. Koelle, G. B.: The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol. Exp. Ther. 103:* 153, 1951.
22. Koelle, G. B. and Friedenwald, J. S.: A histochemical method for localizing cholinesterase activity. *Proc. Soc. Exp. Biol. Med. 70:* 617, 1949.
23. Koelle, G. B. and Gromadzki, C. G.: Comparison of the gold-thiocholine and gold-thiolacetic acid methods for the histochemical localization of acetylcholinesterase and cholinesterases. *J. Histochem. Cytochem. 14:* 443, 1966.
24. Kokko, A.: Histochemical and cytophotometric observations on esterases in the spinal ganglion of the rat. *Acta Physiol. Scand. 66:* suppl. 261, 1, 1965.
25. Lehrer, G. M. and Ornstein, L.: A diazo coupling method for the electron microscopic localization of cholinesterase. *J. Biophys. Biochem. Cytol. 6:* 399, 1959.
26. Lewis, P. R. and Shute, C. D.: Demonstration of cholinesterase activity with the electron microscope. *J. Physiol. (London) 175:* 5P, 1964.
27. Lewis, P. R. and Shute, C. D.: The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. *J. Cell Sci. 1:* 381, 1966.
28. Manolov, S.: Topography and cholinesterase activity of the nerve endings in striated muscles. *Acta Neuroveg. 25:* 427, 1963.
29. Miledi, R.: Electron microscopical localization of products from histochemical reactions used to detect cholinesterase in muscle. *Nature (London) 204:* 293, 1964.
30. Silver, A.: A histochemical investigation of cholinesterases at the neuromuscular junctions in mammalian and avian muscle. *J. Physiol. (London) 169:* 386, 1963.
31. Snell, R. S. and McIntyre, N.: Changes in the histochemical appearance of the motor end plate following denervation. *Brit. J. Exp. Path. 37:* 44, 1956.
32. Tuncbay, T. O.: Histochemistry of esterases of the motor end plates and the lumbar motorneurons of the cat after sciatic neurectomy followed by primary and secondary anastomosis. *Neurology 14:* 657, 1964.
33. Waser, P. G. and Hadorn, I.: Relations of cholinergic receptors to acetylcholinesterase of end plates in denervated muscle. *Bibl. Anat. 2:* 115, 1961.
34. Zacks, S. I. and Blumberg, J. M.: The histochemical localization of acetylcholinesterase in the fine structure of neuromuscular junctions of mouse and human intercostal muscles. *J. Histochem. Cytochem. 9:* 317, 1961.

Journal of Neurochemistry, 1967, Vol. 14, pp. 947 to 954. Pergamon Press Ltd. Printed in Northern Ireland

CHOLINESTERASES AND ESERINE-RESISTANT CARBOXYLIC ESTERASES IN DEGENERATING AND REGENERATING MOTOR END PLATES OF THE RAT

O. ERÄNKÖ and H. TERÄVÄINEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

(*Received* 20 *April* 1967)

KUPFER (1951) was the first to demonstrate histochemically the persistence of cholinestrase activity for about a month in degenerating motor end plates of the rat. Since then numerous publications have appeared on this subject and cholinesterase activity has been reported to persist in the motor end plate long after the degeneration of the motor nerve (BAUER, BLUMBERG and ZACKS, 1962; BERGNER, 1957; BRZIN and MAJCEN-TKAĆEV, 1963; BRZIN and ZAJICEK, 1958; CSILLIK and SAVAY, 1958; GEREBTZOFF and VANDERMISSEN, 1956; GUTH and ZALEWSKI, 1963; HINES, 1960; SNELL and MCINTYRE, 1956 and WASSER and HADORN, 1961).

In all the above-cited studies the total cholinesterase or esterase activity was demonstrated, often assuming acetylcholinesterase (AChE) responsible. However, overwhelming evidence is available of the multiplicity of esterases involved in producing the histochemical reactions with substrates such as α-naphthyl acetate (see ERÄNKÖ, HÄRKÖNEN, KOKKO and RÄISÄNEN, 1964; HÄRKÖNEN 1964; KOKKO 1965). Acetylthiocholine and butyrylthiocholine are more specific substrates in being, as a rule, hydrolysed only by cholinesterases, the former by both acetylcholinesterase (AChE) and nonspecific or butyrylcholinesterase (BuChE) the latter by BuChE only. Discrimination between these two types of cholinesterase activity is readily possible in histochemical systems with the aid of selective inhibitors (see KOELLE, 1963; ERÄNKÖ *et al.*, 1964; ERÄNKÖ and TERÄVÄINEN, 1967). Though admittedly simplified, division of histochemically demonstrable esterase activities into a non-specific type, which can be demonstrated in the presence of potent inhibitors of cholinesterases such as esterine, and either AChE or BuChE has proved valuable for practical purposes (see ERÄNKÖ *et al.*, 1964). Although likely guesses of the proper classification (see DIXON and WEBB, 1964) of the enzymes involved can be made (in the present case AChE = E.C. 3.1.1.7., BuChE = E.C 3.1.1.8., 'non-specific esterases' = E.C. 3.1.1.1. + E.C. 3.1.1.2. + E.C. 3.1.1.6. + others?), it is preferred to use here the less precise terms in describing the histochemical reactions employed.

As demonstrated by many authors, the motor end plate of the striated muscle of the rat contains not only AChE but also BuChE and non-specific esterase (see ERÄNKÖ and TERÄVÄINEN, 1967). While the multiplicity of esterases is well documented in the normal end plate, adequate substrate–inhibitor combinations have been used by amazingly few investigators to discriminate between these enzymes in studying

Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase.

degeneration and regeneration of the end plate after nerve crushing or transection. FILOGAMO and GABELLA (1966) and SCHWARZACHER (1957) used di-isopropylfluorophosphonate (DFP) to inhibit BuChE. However, DFP has been reported to be unreliable in discriminating between AChE and BuChE because of a very narrow safety margin (HOLMSTEDT and SJÖQVIST, 1961; PEARSE, 1960). TUNCBAY (1964) used *N,N*-bis(2-diethylaminoethyl)oxamide bis(2-chlorobenzylchloride) (Mytelase) to avoid interference by BuChE when demonstrating AChE activity. The reliability of her technique is open to doubt, because the selectivity of mytelase as an inhibitor of cholinesterases other than acetylcholinesterase has not been checked in a histochemical system.

It thus appears that all the studies as yet carried out on the degenerating and regenerating end plate leave it more or less uncertain which kind of esterase or cholinesterase activity is involved. In the study to be reported in the present paper, the responses of AChE, BuChE and carboxylic esterases resistant to eserine, to nerve division are described using reliable inhibitors.

MATERIAL AND METHODS

'Permanent' denervation was carried out by removing unilaterally about 1·5 cm of the sciatic nerve from thirty adult rats of the Sprague–Dawley strain near the proximal end of the femur under ether anaesthesia. While regeneration may be possible after such an operation, no evidence of such was observed in the present work. To study the effect of more temporary denervation and especially, re-innervation, the same nerve in another thirty animals was compressed between forceps in the same region. The contralateral intact side served as a control. The animals were killed 1–240 days after the operation. A piece of the tibialis anterior muscle was fixed overnight at 4° in a solution containing 1 vol. or 35% HCHO. (E. Merck AG, Darmstadt), 6 vol. of 2% $CaCl_2$ and 3 vol. of H_2O.

For the demonstration of AChE, acetylthiocholine iodide (Fluka A. G., Buchs, Schweiz) was used as a substrate with 10^{-5} M-tetra-isopropylpyrophosphoramide (iso-OMPA) (L. Light and Co., Colnbrook, England) as a specific inhibitor of BuChE. BuChE was demonstrated with butyrylthiocholine iodide (Fluka A. G., Buchs, Schweiz) as a substrate and 10^{-4} M-1,5-bis-(4-allyl dimethylammoniumphenyl)pentan-3-one di-iodide (284C51) (Burroughs and Wellcome, London) to inhibit AChE. The method used was the GOMORI (1952) modification of the KOELLE (1951) technique. The length of the incubation was 1–7 hr for AChE and 3–8 hr for BuChE. Incubation was performed at 37° and pH 6·0, using free-floating 30 μ thick sections.

General carboxylic esterase activity was demonstrated using α-naphthyl acetate (The British Drug Houses, Poole, England) as a substrate and Fast Blue RR Salt (Allied Chemical Corporation, New York 6 N.Y.) as a coupling agent. To inhibit all cholinesterases, 10^{-5} M-eserine (Physostigmin, E. Merck, A. G. Darmstadt) was used as a rule but higher concentrations were also employed. To discriminate between organo-phosphorus-resistant and -sensitive non-specific esterases, 10^{-5} M-diethyl *p*-nitrophenyl phosphate (E-600) (Mintacol. Bayer, Leverkusen) was employed. The incubation was carried out at room temperature by continuously filtering fresh incubation mixture on the sections, with eserine for 6–20 min and with E-600 for 30–45 min.

In all cases involving inhibitors, the sections were preincubated for 30 min in a substrate-free mixture containing the inhibitor, which was also incorporated in the substrate mixture. Control sections incubated with both iso-OMPA and 284C51, or with eserine alone, were consistently negative when the thiocholines were used as substrates, which indicates that these substrates were hydrolysed only by cholinesterases.

RESULTS

Acetylcholinesterase. In the normal motor end plate (Fig. 1) an intense AChE reaction was observed in the peripheral complex of synaptic folds (F), while the reaction was less intense in the central region of the terminal axon (A). The muscle and the teloglial cells were negative.

In the course of the first 10 days after nerve reaction, a marked decrease was observed in the intensity of the AChE reaction, and the synaptic lamellae, poorly visible in the normal end plate, became more sharply delineated (Fig. 2). Thereafter

the intensity of the AChE reaction weakened slowly. The subneural apparatus shrank, but otherwise only minor morphological changes were observed during the first month after the operation (Fig. 3). Subsequently, the subneural apparatus became slowly more and more deformed, shrank laterally and became longer in relation to its diameter, possibly due to degenerative changes in the muscle fibre (Figs. 4 and 5). However, a weak AChE reaction was observed up to 8 months after the nerve resection (Fig. 5). It should be realized that for this reaction to occur the incubation time had to be lengthened to 7 times that required for the controls, and even so only a weak AChE reaction was obtained. Thus, it is a matter of judgement whether the reaction is considered weak but positive, or essentially negative; we subscribe to the former view. Since there was a distinct difference between the reaction obtained without inhibitor and with 284C51, which rendered the reaction negative, autolysis of the substrate was not responsible.

After crushing of the nerve, the changes observed during the first 2 months were similar to those seen after neurectomy. Thereafter, signs of regeneration appeared, the regenerating end plates showing a recovery in the intensity of the AChE reaction. All end plates exhibited a reaction of normal intensity in 4 months after crushing. However, the impression was gained that, even after 8 months, the regenerated end plates were not morphologically quite similar to the intact ones, the subneural lamellae of the former (Fig. 6) being perhaps somewhat more irregularly arranged than those of the latter, the great majority of which had a regular arrangement of the synaptic folds.

Butyrylcholinesterase. In normal end plates (Fig. 7), the BuChE reaction was approximately of the same order of intensity in the region of the terminal axon (A), in the subneural apparatus (F) and in the teloglial cells (T).

After 'permanent' denervation by nerve resection the intensity of the BuChE reaction became very much weaker but even after 8 months a weak reaction was still observed in the deformed subneural apparatus (Fig. 10). It is of course easier to become convinced of the persistence of the reaction by direct microscopic examination of microscopic slides than from black and white photomicrographs of a single end plate, in which refractile structures may appear dark although they do not, in fact, exhibit any colour. In the course of the first 10 days the decrease in the intensity of BuChE reaction was less striking than that in the AChE activity. The teloglial cells preserved their normal level of BuChE activity. Moreover, new BuChE activity appeared along numerous nerve trunks and fibres during the 4th postoperative day (Fig. 8). This activity was localized in the normally negative Schwann cells of these fibres. The intensity of this neurolemmal activity reached its maximum on about the 10th postoperative day and thereafter it remained elevated for the next 8 months (Fig. 9). During that time the BuChE reaction of the subneural apparatus was hardly visible amongst the strongly reactive Schwann cells (Fig. 9).

During the first 2 months after crushing of the sciatic nerve, the changes observed in the regenerating end plates were similar to those observed after nerve resection; the activity decreased in the end plates and new activity appeared in the Schwann cells. Thereafter, in the course of the next 2 months, the intensity of the BuChE reaction in the end plates normalized, while the Schwann cells gradually lost their elevated BuChE activity (Fig. 11). The motor end plate structure was not as regular as that of the control

Explanations and abbreviations:
A, axon, F, fold complex of the subneural apparatus, T, teloglial cell, N, motor nerve, MEP, motor end plate, G, Schwann cell, FC, fat cell.

FIG. 1.—AChE in a normal end plate. Note the intense reaction in the peripheral subneural fold complex (F), as compared with the central axonal region (A). Acetylthiocholine and iso-OMPA, incubation time 1 hr. ×1365.

FIG. 2.—AChE in an end plate 10 days after transection of the motor nerve. Synaptic folds are visible also in the axonal region. Technique as in Fig. 1. ×1365.

FIG. 3.—AChE in an end plate 1 month after division of the motor nerve. Technique as in Fig. 1. ×1365.

FIG. 4.—AChE in an end plate 8 months after irreversible division of the motor nerve by removal of a segment of it. The AChE reaction is clearly weaker, but the lamellar structure of the subneural apparatus is quite distinct. Acetylthiocholine and iso-OMPA, incubation time 3 hr. ×1365.

FIG. 5.—AChE in two end plates 8 months after transection of the motor nerve by removal of a piece. The weak AChE reaction is exclusively confined to the shrunken subneural apparatus in which the lamellar structure is visible. Neither fragmentation nor granularization can be observed. Acetylthiocholine and iso-OMPA, incubation time 7 hr. ×1365.

FIG. 6.—AChE in an end plate 8 months after degeneration due to crushing of the nerve. Reaction intensity has became normal, but the structure of the subneural apparatus is somewhat irregular compared with the normal end plate of Fig. 1. Technique as in Fig. 1. × 1365.

FIG. 7.—BuChE in a normal end plate. Both axonal (A) and subneural (F) components can be observed, but not so clearly as in AChE reaction in Fig. 1. Teloglial cells (T) are positive. Butyrylthiocholine and 284C51, incubation time 3 hr. ×1365.

FIG. 8.—BuChE in an end plate 36 days after transection of the sciatic nerve. The structure of the subneural apparatus (F) is well preserved. Note the reaction in the Schwann cells (G) along the degenerating nerves, presumably motor. Technique as in Fig. 7. ×1365.

FIG. 9.—BuChE in tibialis anterior muscle 240 days after denervation by removal of a piece of the sciatic nerve. A strong BuChE reaction is observed along the degenerating nerves (N), in the subneural apparatus (MEP) and, though weakly, in the fat cells (FC). Butyrylthiocholine and 284C51, incubation time 8 hr. ×136.

FIG. 10.—BuChE in two end plates 240 days after resection of a piece of the corresponding nerve. A weak BuChE reaction is visible in the subneural apparatus, in which the lamellar structure is seen. Technique as in Fig. 9. ×1365.

FIG. 11.—BuChE 76 days after reversible degeneration by crushing of the sciatic nerve. Nearly normal BuChE reaction is observed in the regenerating end plates. The BuChE reaction along the nerve fibres to the end plates in the Schwann cells (N) is still weakly positive, like the fat cells (FC). Technique as in Fig. 7. ×1365.

FIG. 12.—BuChE in an end plate 240 days after crushing of the sciatic nerve. The intensity of the reaction is normal but the structure is more irregular than that of the normal end plate in Fig. 7. Technique as in Fig. 7. ×1365.

FIG. 13.—Eserine-resistant esterases in a normal end plate. Staining is most intense in the central region of the axon (A), as compared with the peripheral region of the synaptic folds (F), which is the reverse of the distribution of AChE (cf. Fig. 1). Teloglial cells (T) are weakly positive. α-Naphthyl acetate and eserine. ×1365.

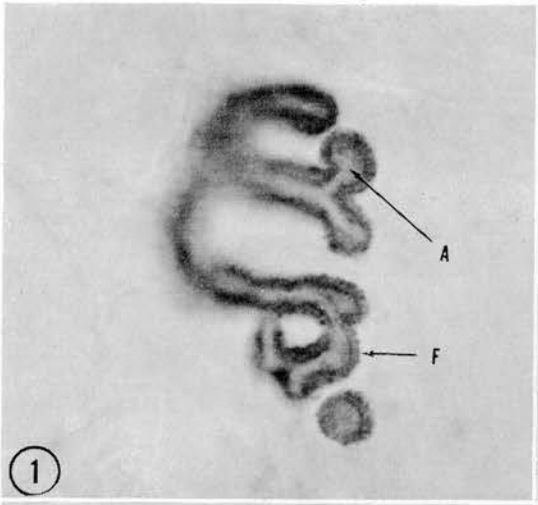
FIG. 14.—Eserine-resistant esterases in end plates 66 days after irreversible division of the motor nerve. The reaction is moderately positive in the motor end plates (MEP) and along degenerating nerves (N). Technique as in Fig. 13. ×136.

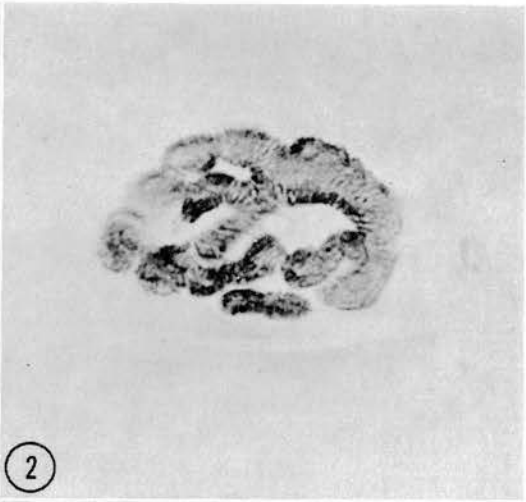
FIG. 15.—Eserine-resistant esterases in end plates 64 days after crushing of the motor nerve. The reaction is intense in the motor end plates (MEP) but quite weak along the motor nerves (N). Technique as in Fig. 13. ×136.

FIG. 16.—E-600-resistant esterases in a normal motor end plate. Differentiation of the different components of the myoneural junction giving this granular reaction is impossible. α-Naphthyl acetate and E-600. ×1587.

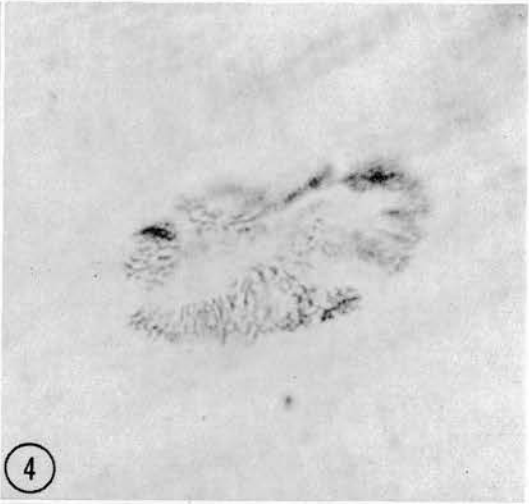
FIG. 17.—E-600-resistant esterases in end plates 34 days after division of the sciatic nerve. Both motor end plates (MEP) and the degenerating motor nerves (N) are positive. Technique as in Fig. 16. ×273.

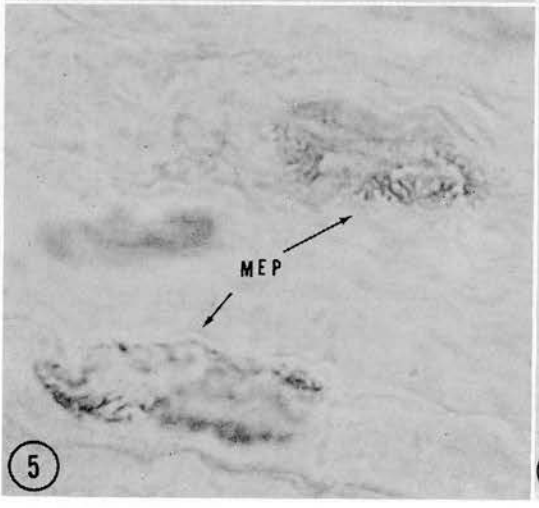
FIG. 18.—E-600-resistant esterases in regenerating end plates 64 days after crushing of the sciatic nerve. Only a weak reaction is observed in the motor end plate. Technique as in Fig. 16. ×136.

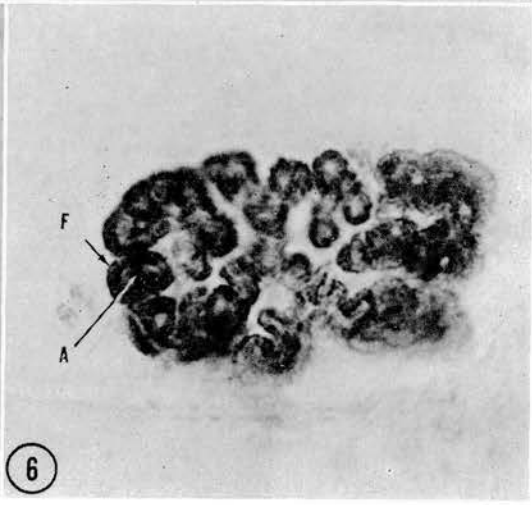

A
F
1

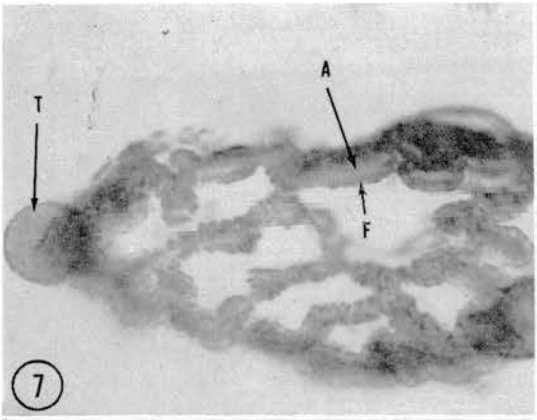

2

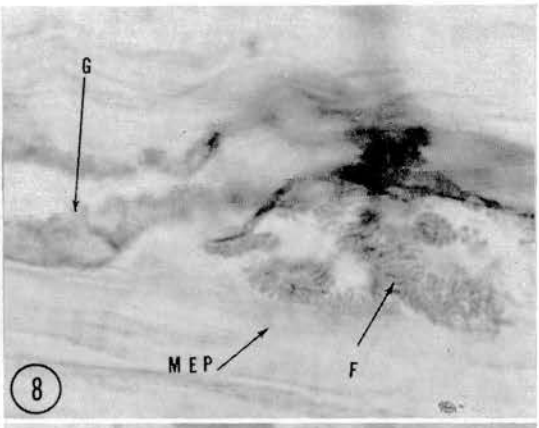

3

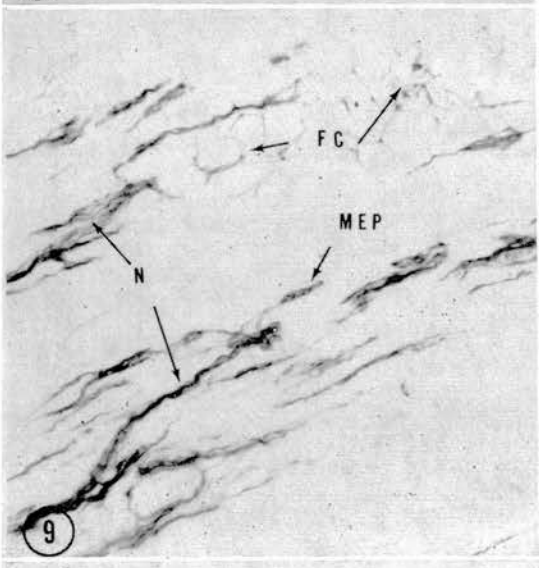

4

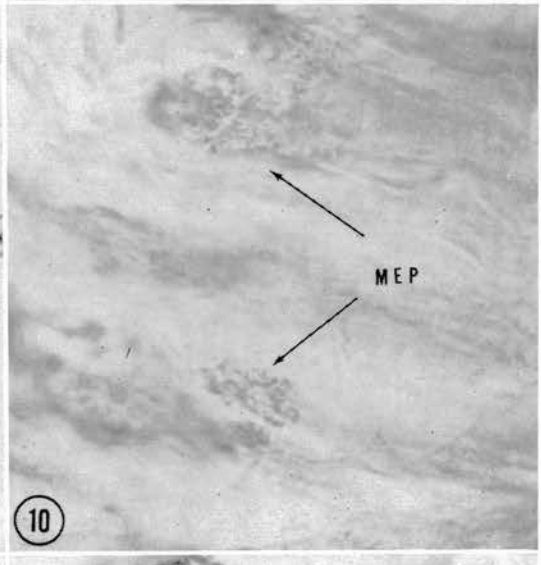

MEP
5

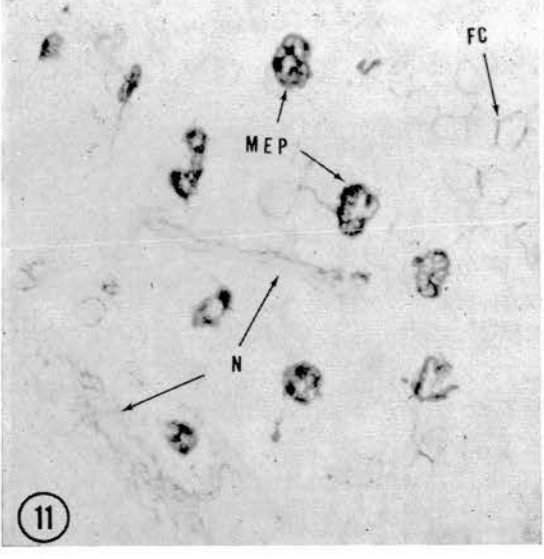

F
A
6

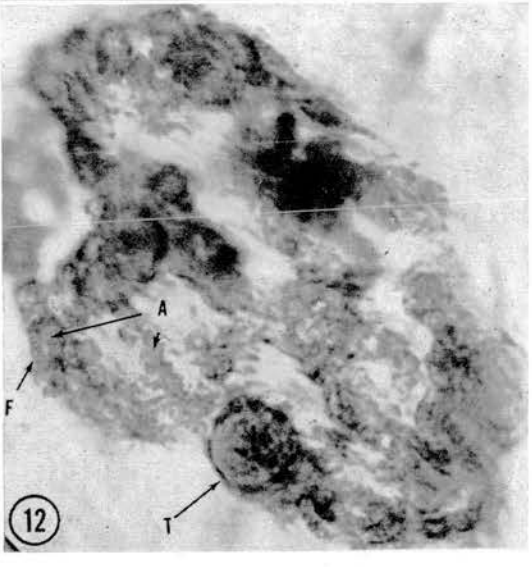

T
A
F
7

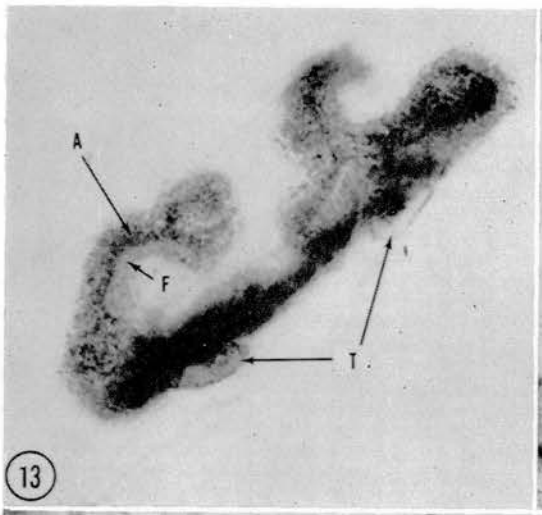

G
MEP
F
8


FC
MEP
N
9

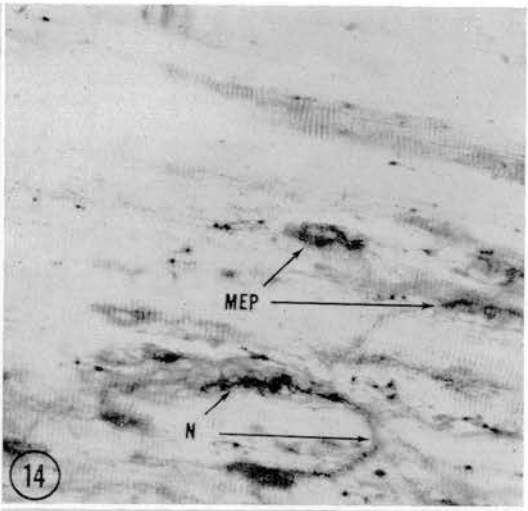

MEP
10


FC
MEP
N
11

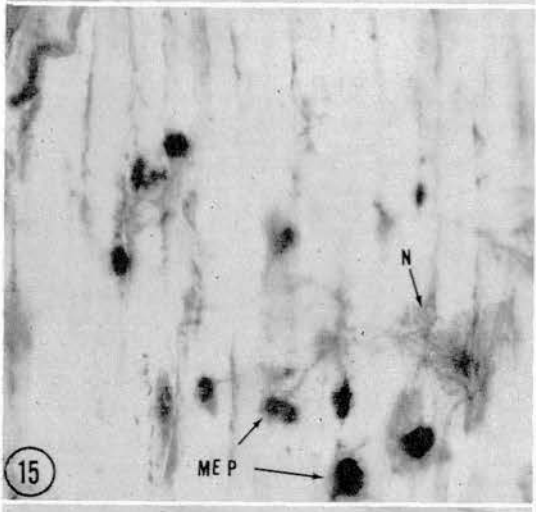

A
F
T
12



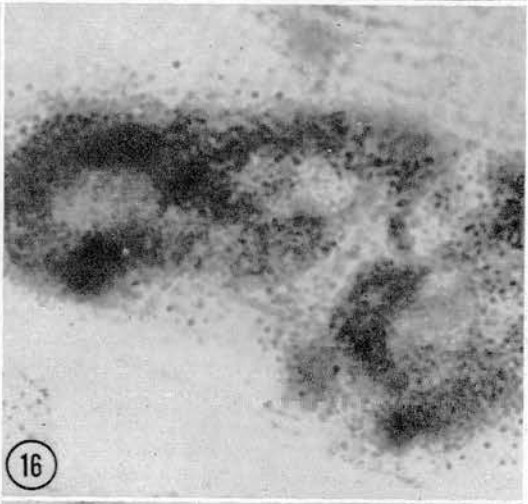
13

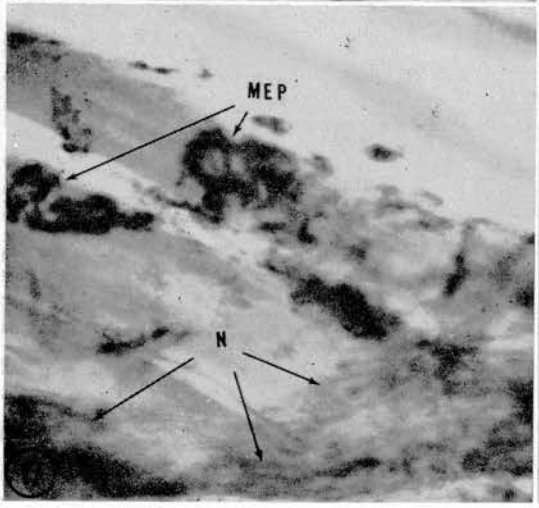


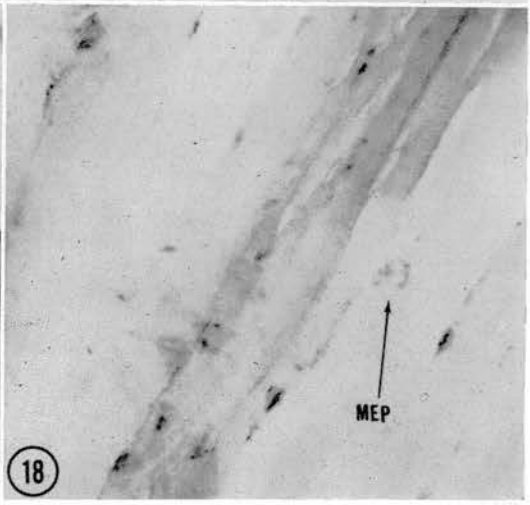
14



15


16





18

side even after 8 months regeneration (Fig. 12), as was also revealed by the AChE reaction.

Non-specific esterases. Esterase activity demonstrable with α-naphthyl acetate and 10^{-5} M-eserine (Fig. 13) was normally localized mainly in the terminal axon (A) but also in the subneural folds (F) and, to a minor degree, in the teloglial cells (T).

This activity was predominantly E-600 sensitive and it had disappeared from the motor end plates by about 3 months after the nerve division. While normal nerves in the muscle were negative, a weak E-600 sensitive activity appeared along the degenerating nerves 7 days after the nerve division (Fig. 14). It seemed to be located in the Schwann cells. This neurolemmal activity was lost 86 days after the operation.

After nerve crushing the intensity of the E-600 sensitive but eserine-resistant esterase reaction of the end plate first decreased. However, it became normal again about 2 months after crushing. An abnormally positive reaction was observed along the motor nerves 2·5 months after crushing (Fig. 15), but this elevated activity was lost before the 4th month after crushing.

Esterase activity resistant to E-600 was present in the motor end plate but its exact localization was impossible to tell (Fig. 16). This activity was absent about 2 months after nerve section. An equal period after nerve crushing, only a faint reaction was observed in the region of the motor end plate (Fig. 18). However, the regeneration of the reaction was completed in 4 months. E-600 resistant activity appeared along the degenerating motor nerves 7 days after the nerve resection. This activity was lost in about 2 months after the operation (Fig. 17) and it was not present along the regenerating nerves thereafter (Fig. 18).

DISCUSSION

About 10 days after removal of a piece of the nerve, the axon terminals have completely disappeared from the myoneural junction and only the subneural apparatus and the teloglial cells remain (COUTEAUX, 1955; GUTH 1956; REGER, 1955). However, all authors agree that the myoneural junction preserves its cholinesterase activity long after neurectomy (see earlier), thus demonstrating that this residual activity is localized in the subneural apparatus. Long periods of persisting activity were also previously reported in the rat: according to some 3 months (GEREBTZOFF and VANDERMISSEN, 1956), and to others about 6 months (CSILLIK and SAVAY, 1958). In the present study, some activity was observed up to 8 months after resection of a piece of the nerve innervating the muscle. Cholinesterase activity had disappeared from the end plates of the mouse after 77 (BAUER, BLUMBERG and ZACKS, 1962) or 120 days (WASER and HADORN, 1961) and from those of the guinea pig after 45 days (SNELL and MCINTYRE, 1966) or after 66 days (BERGNER, 1957). However, different authors may well have judged the reactions, positive or negative, in different ways. Rapid re-innervation of the motor end plate has been reported in several species. In the rat the ChE activity of the end plate has been found to be normal at 21 days (SCHWARZACHER, 1957), 30 days (CSILLIK, 1965) or 120 days (HINES, 1960) after nerve crushing; in the mouse a period of 30 days has been reported (WASER and HADORN, 1961), in the guinea pig and the hen it has been 30–35 days (FILOGAMO and GABELLA, 1966), and in a bigger animal, such as the cat, about 90 days (TUNCBAY, 1964).

CSILLIK and SAVAY (1958) and CSILLIK (1965) reported fragmentation of the histochemical cholinesterase reaction—due in their study to both AChE and BuChE—in

the subneural apparatus during the first 30–90 postoperative days and still later, after 90–180 days, granularization of the reaction in the subneural apparatus. We were unable to see such granularization and fragmentation, which Csillik (1965) interpreted as a sign of inability of the myoneural junction for simple reinnervation. Previous observations by other authors suggest, indeed, that recovery of motor function can be achieved in striated muscles after a delay in suturing as long as 11 months (Guth, 1956) and that there are relatively minor morphological changes in the end plate after 9 months of atrophy (Gutman and Young, 1944). In fact, original end plates are reported to be re-innervated even after 17 months of atrophy (Gutman and Young, 1944), though simultaneously also new end plates can be formed (Csillik, 1965; Guth and Zalewski, 1963; Gutman and Young, 1944; Gwyn and Aitken, 1966).

In the present study, the Schwann cells of the divided nerves became reactive for BuChE from the 4th postoperative day onwards. This activity was lost again only if regeneration of the motor end plate occurred, as was clearly indicated by the recovery in it of the activities of AChE, BuChE and eserine-resistant esterase activity. In contrast to these observations Csillik (1965) reported that the cholinesterase activity which appeared in the nerve fibres from the 28th postoperative day onwards disappeared during the 4th month, even without regenerative alterations in the motor end plate. The increase of the BuChE reaction in the distal part of the divided nerve is closely timed with the destructive changes in the myelin of the motor axons (see Guth, 1956; and references therein). The Schwann cells may indeed serve as guiding tracks to the regenerating axons as was already proposed by Cajal (1933). In fact, no regenerative changes have been observed in the nerves without the Schwann cells (Guth, 1956).

The persistence of abnormal BuChE activity in the Schwann cells, after the phase of myelin destruction, as long as regeneration proceeds might have some bearing on the regeneration process. Such an idea was also suggested by Hines (1960), who used acetylthiocholine and eserine to demonstrate cholinesterase activity, which he believed to be due to AChE. Our work, supported by the observations of Bergner (1957), clearly shows that BuChE and not AChE is responsible.

Filogamo and Gabella (1966) reported AChE activity in the sarcoplasm of the muscle fibre 10 days after motor nerve division. Brzin and Majcen-Tkácev (1963) reported BuChE and, to a lesser amount, AChE activity as 'broken bands or twigs in the muscle fibres probably due to fragments of subterminal nerve endings and/or terminal arborizations'. No staining was observed in the present study in the muscle fibres even after incubation for up to 8 hr. The staining observed by the above authors may have been located in the Schwann cells but interpreted to be in the muscle fibres, or else the muscular reaction was due to diffusion.

In the present study regenerative changes were seen in the myoneural junction 2 months after the nerve crushing. As in many previous studies (Couteaux, 1955; Csillik, 1965; Csillik and Savay, 1958; Filogamo and Gabella, 1966; Hines, 1960), it was clear that all original end plates became re-innervated: the intensities of the AChE, BuChE and other esterase reactions returned to normal, no degenerating end plates were seen and no BuChE-positive Schwann cells were observed along the motor nerves. As in the study by Hines (1960) on cat end plates, the regeneration of rat end plates was found in the present study to be completed in 4 months. This is a

considerably longer period than that of 3 weeks reported by SCHWARZACHER (1957) and that of 1 month reported by CSILLIK (1965) in the rat. Such differences may be explained by differences in the severity of the crushing, in the distance from the severed part to the end plate, and in the criteria used to follow the regeneration process. We followed not only esterase reactions of the end plate but also the changes in the BuChE activity in the Schwann cells of the motor nerve.

In our study, the regenerated subneural apparatus did not quite recover the regular appearance of the normal end plate even 8 months after crushing of the nerve, in contrast to previous reports (CSILLIK and SAVAY, 1958; FILOGAMO and GABELLA, 1966; HINES, 1960). This difference may be due to the relatively long period in our study before re-innervation took place (GUTMAN and YOUNG, 1944).

TUNCBAY (1964) reported in the cat that the BuChE activity regenerates in the end plates faster than the AChE activity after nerve division and primary suturing. In TUNCBAY's series there was also a time difference between the reaction obtained with α-naphthyl acetate and that due to thiolacetic acid, the former of which regenerated fastest of all esterases whilst the latter did not regenerate at all. In the present study AChE, BuChE and eserine-resistant esterase activities all regenerated at the same rate, but the E-600 resistant esterase activity regenerated more slowly. All types of esterases were completely regenerated in 4 months.

It is obvious from previous studies and the present work that degeneration and regeneration of the motor axon is the underlying cause for the decrease and subsequent rise in the histochemically demonstrable AChE, BuChe and other esterase activities of the end plate. Thus, these enzyme activities reflect the destruction and reactivation phases of the metabolic and synaptic functions of the end plate. However, it still remains open to question, even after this present study, why AChE and BuChE activities persist in the degenerating motor end plate. While 'old' molecules of enzyme remaining in the subneural apparatus and disappearing very slowly during degeneration may be responsible, the subneural apparatus may also be capable of synthesizing new enzymes after their disappearance from the motor axon. If the latter alternative is true, perhaps the enzymes indeed reflect the capacity of the myoneural junction for re-innervation, as was proposed by CSILLIK (1965).

SUMMARY

Distribution of acetylcholinesterase (AChE), non-specific cholinesterase (BuChE) and eserine-resistant 'non-specific esterases' was studied in the motor end plate of the tibialis anterior muscle of the rat after 'permanent' denervation by removal of a piece of the sciatic nerve and after temporary denervation by compression of the nerve. Acetylthiocholine, butyrylthiocholine and α-naphthyl acetate were used as substrates together with selective inhibitors.

After removal of a piece of nerve AChE activity was observed to weaken with time in the subneural apparatus of degenerating motor end plates for up to 8 months. Decreased BuChE activity was also detected up to 8 months in the subneural apparatus and the teloglial cells. From the 4th postoperative day up to 8 months, distinct BuChE activity was found in the normally negative Schwann cells. Eserine-resistant esterase activity decreased but was demonstrable in the subneural apparatus in degenerating motor end plates for about 3 months, and it appeared from the 7th postoperative day along the degenerating motor nerve fibres.

During the first 2 months the changes observed after reversible denervation by crushing of the nerve were similar to those seen after nerve division. During the next 2 months thereafter, the intensity of the AChE, BuChE and other esterase activities became normal at about the same rate. Parallel with the recovery of the esterase reactions in the end plates, the Schwann cells gradually lost their abnormally elevated BuChE activity. All reactions were normalized in the motor end plate as well as in the Schwann cells in 4 months after the operative interference.

REFERENCES

BAUER W. C., BLUMBERG J. M. and ZACKS S. I. (1962) *Proc. 4th Int. Congr. Neuropath. Munich*, p. 16. Georg Thieme, Stutttgart.

BERGNER A. D. (1957) *Brit. J. exp. Path.* **38,** 160.

BRZIN M. and MAJCEN-TKÁCEV Z. (1963) *J. Cell. Biol.* **19,** 349.

BRZIN M. and ZAJICEK J. (1958) *Nature (Lond)* **181,** 626.

CAJAL S. (Editor) (1933) *Histology*, Bailliére, Tindall and Cox, London.

COUTEAU R. (1955) *Int. Rev. Cytol.* **4,** 335.

CSILLIK B. (Editor) (1965) *Functional structure of the postsynaptic membrane in the myoneural junction.* Akadémiai Kiadó, Budapest.

CSILLIK B. and SÁVAY G. (1958) *Acta neuroveg. Wien* **19,** 41.

DIXON M. and WEBB E. C. (1964) *Enzymes.* Second edition. Longmans, London.

ERÄNKÖ O., HÄRKÖNEN M., KOKKO A. and RÄISÄNEN L. (1964) *J. Histochem. Cytochem.* **12,** 570.

ERÄNKÖ O. and TERÄVÄINEN H. (1967) *J. Histochem. Cytochem.* In press.

FILOGAMO G. and GABELLA G. (1966) *Acta anat. (Basel)* **63,** 199.

GEREBTZOFF M. A. and VANDERMISSEN L. (1956) *Ann. Histochim.* **1,** 221.

GOMORI G. (Editor) (1952) *Microscopic Histochemistry, Principles and Practice.* The University of Chicago Press, Chicago.

GUTH L. (1956) *Physiol. Rev.* **36,** 441.

GUTH L. and ZALEWSKI A. A. (1963) *Exp. Neurol.* **7,** 316.

GUTMAN E. and YOUNG J. Z. (1944) *J. Anat. (Lond.)* **78,** 15.

GWYN D. G. and AITKEN J. T. (1966) *J. Anat. (Lond.)* **100,** 111.

HINES M. (1960) *Anat. Rec.* **138,** 283.

HOLMSTEDT B. and SJÖQVIST F. (1961) *Biol. anat (Basel)* **2,** 1.

HÄRKÖNEN M. (1964) *Acta physiol. scand.* **63,** Supp. 237.

KOELLE G. B. (1951) *J. Pharmacol. exp. Ther.* **103,** 153.

KOELLE G. B. (1966) *Cholinesterases and anticholinesterase agents. Handbuch der Experimentellen Pharmakologie. Vol.* 15, p. 187. Springer-Verlag, Berlin.

KOKKO A. (1965) *Acta physiol. scand.* **66,** Supp. 261.

KUPFER C. (1951) *J. cell. comp. Physiol.* **38,** 469.

PEARSE A. G. E. (1960) *Histochemistry, Theoretical and applied.* J. & A. Churchill Ltd., London.

REGER J. F. (1955) *Anat. Rec.* **122,** 1.

SCHWARZACHER H. G. (1957) *Acta anat. (Basel)* **31,** 507.

SNELL R. S. and MACINTYRE N. (1956) *Brit. J. exp. Path.* **37,** 44.

TUNCBAY T. O. (1964) *Neurology (Minneap.)* **14,** 657.

WASER P. G. and HADORN I. (1961) *Biol. anat. (Basel)* **2,** 155.

Histochemical Journal, **2** (1970), 479–489

Light and electron microscopic histochemical observations on cholinesterase-containing sympathetic nerve fibres in the pineal body of the rat

OLAVI ERÄNKÖ, LEENA RECHARDT, LIISA ERÄNKÖ
and AULIKKI CUNNINGHAM

Department of Anatomy, University of Helsinki, Siltavuorenpenger Helsinki, Finland

Received 25 May and in revised form 28 August 1970

Synopsis. Pineal glands of adult albino rats were examined histochemically using, first, formaldehyde-induced fluorescence to study monoamines and, second, copper thiocholine or copper ferrocyanide methods to study acetylcholinesterase and non-specific cholinesterase by light and electron microscopy. Cholinesterase was determined quantitatively by a constant pH titration assay.

Fluorescent and acetylcholinesterase-positive nerve nets formed identical patterns. Non-specific cholinesterase was observed only in nerve trunks outside the pineal. Bilateral removal of superior cervical ganglia resulted in complete disappearance of fluorescence and acetylcholinesterase from nerve fibres. Electron microscopically, acetylcholinesterase was found on sympathetic axons containing small granular vesicles. Quantitative cholinesterase determinations suggested that the pineal activity was mainly due to acetylcholinesterase. Comparison of the incubation times required for equal histochemical acetylcholinesterase reactions suggested that the activity of the sympathetic nerve fibres in the pineal is of the same order of magnitude as that in the nerve fibres of the iris.

Introduction

The sympathetic nerve fibres originating from the superior cervical ganglia supply the main innervation of the pineal body; these nerve fibres and accompanying blood vessels reach the pineal via the epiphyseal stalk (Kappers, 1965). Although some habenular and caudal commissural fibres have been said to connect the pineal body to the brain stem, these fibres are supposed to be aberrant (Kappers, 1960).

The pineal body of several animal species, including the rat, has been reported to be devoid of histochemically detectable cholinesterase activity (Arvy, 1961; Arstila, 1966). Biochemically, a weak activity has been demonstrated in the pineal bodies of the sheep and the ox (Thiéblot *et al.*, 1966; La Bella & Shin, 1968). La Bella & Shin (1968) estimated the acetylcholinesterase content of the bovine pineal body to be about 7% of the activity of the brain tissue.

On the basis of pharmacological observations, a parasympathetic, cholinergic mechanism

was recently proposed in the pathway mediating the light-dependent changes of the pineal serotonin metabolism of the rat from the eye through the superior cervical ganglia to the pineal (Wartman *et al.*, 1969).

The present study was undertaken to examine histochemically whether the pineal body of the rat would show cholinesterase activity. Light and electron microscopical histochemical methods and biochemical enzyme determinations were applied to pineals of normal and sympathectomized rats.

Materials and methods

Formaldehyde-induced fluorescence

After decapitation of fifteen adult rats, the pineal bodies were removed and rapidly frozen by immersion in propane precooled in liquid nitrogen. They were then dried for 7 days *in vacuo* at −45°C and exposed for 1–2 hr at 50–80°C in formaldehyde gas generated from paraformaldehyde powder, which had been equilibrated in an atmosphere of 60% humidity (see Eränkö, 1967*b*). They were then embedded in Epon and sections were cut with a glass knife at 2 μm. The fluorescence was photographed with a fluorescence microscope equipped with an HBO-200 high-pressure mercury lamp and Schott BG 12 and OG 1 filters.

Cholinesterases

Sixty adult male albino rats, descendants of the Sprague–Dawley strain, were used. The rats were perfusion-fixed via the left heart ventricle under artificial oxygen and carbon dioxide respiration with (1) 2% formaldehyde–1% glutaraldehyde mixture in 0.1 M phosphate buffer, pH 7.2, or (2) 3.5% formaldehyde in Krebs–Ringer glucose, pH 6.9 (Eränkö *et al.*, 1967). After 15 min perfusion the pineal bodies were excised and fixation was prolonged by further immersion in the respective fixatives for 20 min. Alternatively, the glands were immersed directly in the ice-cold fixatives. Tissues fixed with the formaldehyde-glutaraldehyde mixture were rinsed in 0.1 M phosphate buffer overnight at 4°C; those fixed with 3.5% formaldehyde were rinsed likewise overnight but in distilled water at 4°C (Robinson, 1969).

For light microscopy, 10 μm sections were cut with the freezing microtome. Two modifications of Koelle's (1951) thiocholine technique for localizing cholinesterase were used: the copper thiocholine method as presented by Gomori (1952) and the copper ferrocyanide method introduced by Karnovsky & Roots (1964).

For electron microscopy, the fixed and rinsed pineal bodies were chopped into small pieces with a razor blade and incubated at pH 5.0, 5.5, 6.0 or 6.5 according to Karnovsky & Roots (1964). The incubation times varied from 10 min to 12 hr at 25 or 37°C. Citrate, used as a chelator in the Karnovsky–Roots (1964) modification, was replaced by 20 mM tartrate in some experiments, as recommended by Kokko *et al.* (1969). The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide (Fluka AG, Buchs). As inhibitors, the following substances were used: 10^{-5} M eserine, 10^{-4}, 10^{-5} and 10^{-6} M iso-OMPA (tetra-isopropylpyrophosphoramide; purchased from L. Light & Co. Ltd, Colnbrook, England) and 10^{-5}M 284C51 (1,5-bis(4-allyl-dimethylammoniumphenyl)pentan-3-one dibromide; purchased from Wellcome Research Laboratories, England). Some incubations were performed without substrate in the incubation medium. The inhibitors were dissolved in the substrate-free preincubation mixture, in which the specimens were treated for 30 min, and in the substrate-containing incubation mixture.

Sections intended for examination in the light microscope were mounted in glycerol gelatine or, after dehydration, in Entellan (E. Merck). For electron microscopy, the incubated tissue pieces were rinsed in four changes of either maleate buffer or distilled water for 1 hr. In other experiments the incubation was stopped by addition of 5% osmium tetroxide (Robinson, 1969). After refixation in phosphate buffered 1% osmium tetroxide for 30 min the pieces were dehydrated and embedded in Epon. Ultra-thin sections were viewed and photographed unstained with an AEI 801 or a Philips EM 300 electron microscope operated at 40, 60 or 80 kV.

Biochemical assays

For each assay the pineal bodies of eighteen rats were pooled for homogenization. The values are mean values of three assays.

Cholinesterase activity was measured quantitatively by a method in which the acid liberated by enzymatic hydrolysis is automatically titrated with 0.005N sodium hydroxide (Holmstedt *et al.*, 1962). The enzyme activity was expressed in terms of nanomole (nM) of acid liberated per min per mg wet weight of fresh tissue, under the standardized conditions of assay (25°C, pH 8). The substrate used was acetylcholine iodide (Sigma Chemical Company, St Louis, Missouri). To obtain approximate estimates of both total and specific acetylcholinesterase activity, the titrations were first made without inhibitor and then a few minutes after addition of 284C51 to make a 10^{-5} M solution, so as to inhibit acetylcholinesterase. Previous experience had shown that this competitive inhibitor quickly causes disappearance of the activity which it inhibits, thus rendering longer equilibration periods unnecessary.

Sympathectomy

Both superior cervical sympathetic ganglia were removed from five adult male rats. After 10 days the rats were killed and cholinesterases were examined as described above for light microscopy.

Results

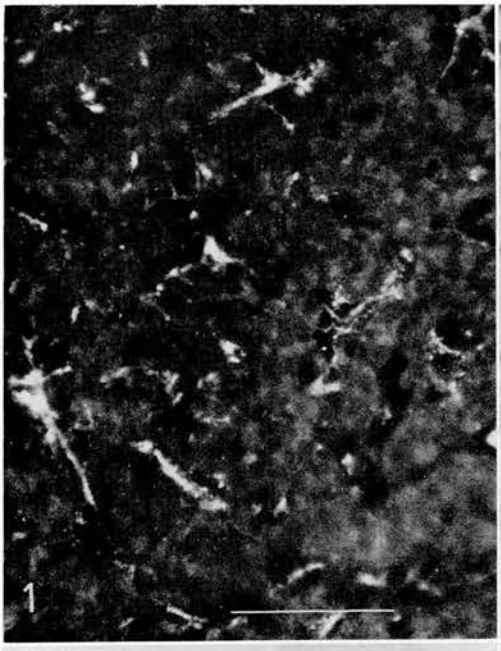
Distribution of formaldehyde-induced fluorescence

Fig. 1 shows the distribution of fluorescence in a pineal gland freeze-dried and exposed to formaldehyde vapour to convert catecholamines and indoxylamines into fluorescent compounds. The background fluorescence was yellowish and faded rapidly on exposure to ultraviolet light, while the more intensely fluorescent nerve fibres appeared greenish. Trunks of fluorescent fibres ramifying in the same manner as in Fig. 1 were seen throughout the pineal gland.

After bilateral removal of the superior cervical ganglia, all fluorescent nerve fibres disappeared.

Light microscopic distribution of cholinesterases

The distribution of acetylcholinesterase in a formalin-fixed pineal as demonstrated by the copper thiocholine technique (Koelle, 1951; as given by Gomori, 1952) using acetylthiocholine as a substrate, and 10^{-5} M or 10^{-6} M iso-OMPA as an inhibitor of non-specific cholinesterase is shown in Fig. 2. Comparison with Fig. 1 indicates that the adrenergic nerve net demonstrated by the fluorescence method and the nerve net exhibiting a positive acetylcholinesterase reaction show identical patterns of coarse and fine fibres. Bilateral removal of the superior cervical


1


2


3


4

ganglia was followed by complete disappearance of acetylcholinesterase-positive fibres, as is evident from Fig. 3.

Essentially similar results were obtained with the method of Karnovsky & Roots (1964), as far as the distribution of the reaction was concerned, while the development of the reaction with this method occurred much more rapidly than that obtained with the Gomori (1952) procedure (see below) in other sections of the same gland.

Non-specific cholinesterase activity demonstrated with butyrylthiocholine and 10^{-5} M 284C51 in formalin-fixed pineal gland was limited to nerve trunks in and outside the capsule, while the main pineal parenchyma was entirely devoid of activity (Fig. 4). The same results were obtained with the method of Karnovsky & Roots (1964).

After denervation, the capsular nerve trunks still exhibited a clear activity of non-specific cholinesterase. Moreover, activity was now observed in the nerve trunks inside the pineal gland, which were not reactive in the controls.

Sections incubated without substrate or preincubated and incubated with 10^{-5} M eserine or with both 10^{-5} M (or stronger) iso-OMPA and 10^{-5} M 284C51 were always devoid of visible reaction.

Estimation of cholinesterase activities

Enzyme activities of the pineal gland and the superior cervical ganglion are compared in Table 1. It can be seen that the acetylcholinesterase activity of the pineal is about 2% of that in the superior cervical ganglion. Actually, the activity of the pineal homogenate was so low that the observed rate of hydrolysis with it was only about three times that without homogenate (spontaneous hydrolysis).

The activity of non-specific cholinesterase was weaker than that of acetylcholinesterase in the pineal, while the non-specific cholinesterase activity of the sympathetic ganglion com-

Table 1. Comparison of cholinesterase activities in the pineal gland and the superior cervical ganglion of the rat. The activities are expressed in terms of nM of acid liberated by mg of fresh tissue per min

Tissue	*Acetylcholinesterase*	*Non-specific cholinesterase*
Pineal gland	0.5	0.2
Superior cervical ganglion	21.3	32.0

Figure 1. Formaldehyde-induced fluorescence in the pineal of a normal rat. Fluorescence is seen both in the nerve fibres and in the pineal cells. Length of the calibration line 100 μm.

Figure 2. Acetylcholinesterase in the pineal of a normal rat. Formaldehyde fixation. Copper thiocholine method (Gomori, 1952), preincubation with 10^{-6} M iso-OMPA and incubation with acetylthiocholine as substrate and 10^{-6} M iso-OMPA as inhibitor of non-specific cholinesterase. Note the similarity of the nerve fibre patterns with those in Fig. 1. Length of the calibration line 100 μm.

Figure 3. Non-specific cholinesterase in the pineal of a normal rat. Method as in Fig. 2 but preincubation with 10^{-5} M 284C51 and incubation with butyrylthiocholine as a substrate and 10^{-5} M 284C51 as inhibitor of acetylcholinesterase. The reaction is limited to nerve trunks outside the main gland, which is non-reactive. Length of the calibration line 100 μm.

Figure 4. Acetylcholinesterase in the pineal of a rat whose superior cervical ganglia were bilaterally removed 10 days before killing. Method as in Fig. 2. Note complete disappearance of the reaction. Length of the calibration line 100 μm.

prised more than one-half of the total activity towards acetylcholine. It should be noted that the total acetylcholine-splitting activity was here divided into acetylcholinesterase and non-specific cholinesterase activity solely on the basis of the effect of 284C51 on the hydrolysis of acetylcholine. This method is not very accurate, especially in view of the low overall activity towards acetylcholine. However, clear loss of most activity after addition of 284C51 may suffice to suggest that the pineal cholinesterase activity is mainly due to acetylcholinesterase.

Any figure of enzyme activity expressed in terms of weight of fresh tissue reflects both the density of the reacting tissue component and the activity level of the enzyme in this component. Since the nerve net of the pineal gland, which was the only tissue exhibiting acetylcholinesterase activity, was relatively scanty, the low activity can be understood as compared with the sympathetic ganglion, the acetylcholinesterase activity of which is present in both the dense network of nerve fibres and the cytoplasm of the nerve cell bodies. The quantitative assay, therefore, does not allow comparison of the intensity of cholinesterase activity in individual axons.

Table 2. Comparison of the lengths of incubation times required for optimum light microscopic acetylcholinesterase reactions in formaldehyde-fixed pineal, iris and sympathetic ganglion of the rat

Tissue	*Incubation time (hr)* *Karnovsky–Roots method*	*Koelle–Gomori method*
Ganglion	0.5	2
Pineal	2	18
Iris	2	18

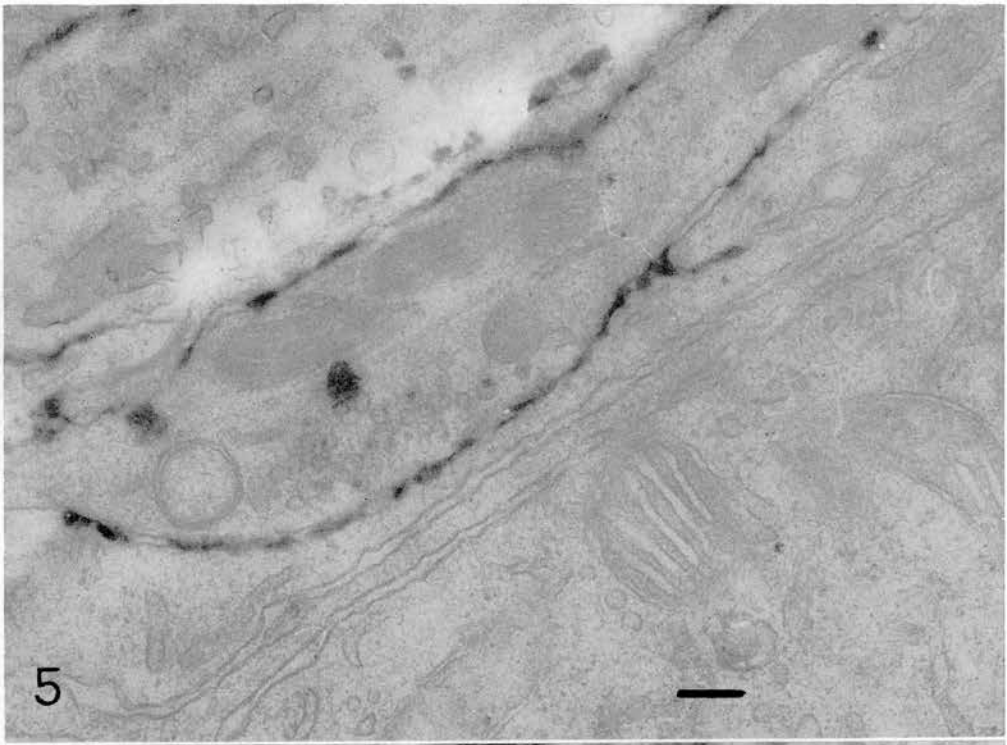
Since such a comparison is of crucial importance from the point of view of the present work, the length of incubation times required to obtain an approximately equal histochemical nerve fibre reaction was determined with the two histochemical methods for the superior cervical ganglion, the pineal gland and the iris. The results are shown in Table 2. The fibres of the ganglion exhibited a reaction much more rapidly than those of the pineal or the iris, which, on the other hand, needed an approximately equal incubation time. In this respect, the results obtained with the Gomori (1952) method agreed with those obtained with the Karnovsky–Roots method (1964), although the latter produced a visible reaction much more quickly.

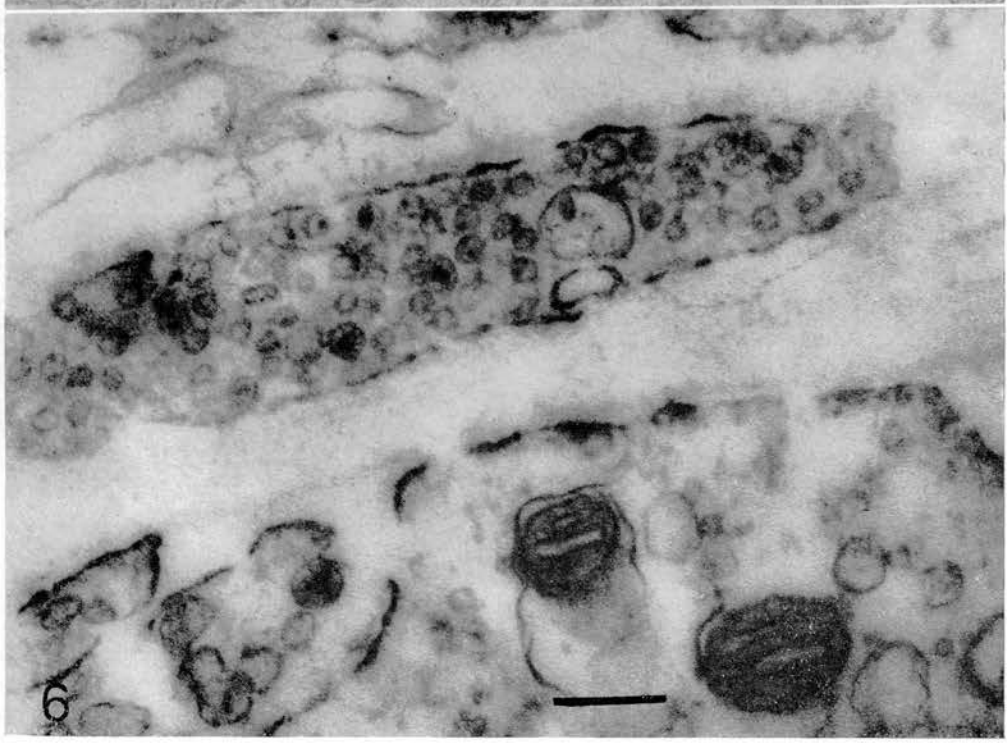
Electron microscopic observations

The same main result was obtained using either formaldehyde or glutaraldehyde fixation: a positive acetylcholinesterase reaction was observed only in association with all pineal axons,

Figure 5. Distribution of acetylcholinesterase in the pineal gland. Fixation in formaldehyde-glutaraldehyde mixture. Preincubation with 10^{-5} M iso-OMPA and incubation with acetylthiocholine and 10^{-5} M iso-OMPA according to Karnovsky & Roots (1964) for 1 hr at pH 6.5. The reaction product around the axon containing small granular vesicles is situated between the axon membrane and the cell membrane. The length of the calibration line is 200 nm (2000 Ångström units).

Figure 6. Distribution of acetylcholinesterase in the pineal gland. Fixation in formaldehyde. Technique as in Fig. 5 but incubation for 10 min at pH 6.0. The reaction is visible in the axon membrane and in the cell membrane of the pinealocyte (below). The length of the calibration line is 200 nm (2000 Ångström units).


5


6

which contained numerous 'empty' synaptic vesicles about 400 Å in diameter and several 'dense-cored' vesicles of about the same size; occasionally, larger vesicles about 1000 Å in diameter were also found amongst the smaller ones.

However, some differences were registered between results obtained with the two fixatives: (a) *Formaldehyde-fixed pineals.* Because of the poor penetration of the reagent, the reaction was limited to a narrow band in the periphery of the small piece of tissue. In all specimens, the reaction was observed *on* the axons in the axon membrane. The fine copper ferrocyanide precipitate formed dark lines which sharply marked the axon membrane (Fig. 6). Sometimes such a reaction was also found on the pineal cell membrane near a reactive axon, while similar cells elsewhere were non-reactive.

(b) *Glutaraldehyde-fixed pineals.* The reaction was likewise observed on axon profiles and their varicosities, both in the perivascular space and close to the pineal cells (Fig. 5). However, the reaction was not continuous but in the form of a chain of fine granules of the reaction product; these granules were situated *outside* the axon membrane, between it and the neighbouring Schwann cell membrane. The pineal cell membrane was always devoid of reaction product, even if close to a reactive axon.

Preincubation and incubation in the presence of 10^{-5} M 284C51 always caused a complete loss of electron microscopically discernible activity in the pineal body.

Discussion

The fluorescence observed in the pineal after exposure to formaldehyde vapour showed two components: a yellow, rapidly fading, parenchymal fluorescence and a more intense and a more stable, greenish nerve fibre fluorescence. This agrees well with previous fluorescence histochemical studies on the pineal showing that 5-hydroxytryptamine is present in the pineal cells, while the nerve fibres contain both noradrenaline, which is the normal sympathetic transmitter, and 5-hydroxytryptamine, which is apparently taken up from the parenchymal pineal cells (Owman, 1964). The previous observation that bilateral removal of the superior cervical ganglion causes a complete disappearance of the fluorescent nerve fibres from the pineal (Owman, 1964), was also confirmed in the present study.

The density and patterns of nerve fibres exhibiting formaldehyde-induced fluorescence were essentially similar to those of the acetylcholinesterase-positive nerve fibres. Indeed, the similarity was so striking that it in itself suggested that the fluorescence reaction and the acetylcholinesterase reaction are located in the same site. However, although the patterns formed by cholinesterase-positive and fluorescent fibres are very similar in the rat iris, consecutive or simultaneous demonstration of formaldehyde-induced fluorescence and acetylcholinesterase in a single iris preparation has indicated that many amine-containing nerve fibres are distinct from the cholinesterase ones (Eränkö & Räisänen, 1965). Similar direct comparison of amine fluorescence and cholinesterase would obviously be desirable also in the case of the pineal. Our preliminary efforts to do so have been discouraging, because exposure to formaldehyde sufficiently long to demonstrate the amine fluorescence in the nerve fibres also abolished the acetylcholinesterase reaction.

Non-specific cholinesterase was in the present study clearly demonstrated in nerve trunks about to enter the pineal gland. These nerve trunks were probably included in the chemical determinations. It is interesting to note that after bilateral sympathetic denervation a positive reaction was observed in the normally negative finer nerve fibres inside the pineal, while

denervation rendered the acetylcholinesterase reaction entirely negative. This is possibly due to a mechanism resembling that in the motor nerves of the striated muscle after division of the motor nerve: normally non-reactive preterminal fibres exhibit after decentralization an intense reaction for non-specific cholinesterase, which is localized in the Schwann cells (Eränkö & Teräväinen, 1967).

A variety of acetylcholinesterase techniques were used in the present study, with essentially similar results: the copper thiocholine method (Koelle, 1951; Gomori, 1952), the copper ferrocyanide method (Karnovsky & Roots, 1964) and the modification by Kokko *et al.* (1969) were applied to both frozen and unfrozen pieces of specimens fixed in formaldehyde or glutaraldehyde, and examined by two independently working groups using both light and electron microscopy. Under all these conditions, the reaction obtained after preincubation and incubation in the presence of 10^{-5} M or 10^{-6} M iso-OMPA (using acetylthiocholine as substrate) and which is inhibited by 10^{-5} M 284C51 can be considered to be due to specific acetylcholinesterase (see Eränkö *et al.*, 1964). This reaction was limited to nerve fibres inside the pineal gland, including very fine ones, while no reaction was obtained inside the gland when butyrylthiocholine was used as a substrate for selective demonstration of non-specific cholinesterase, together with 10^{-5} M 284C51 in the preincubation and incubation mixtures to inhibit acetylcholinesterase.

The acetylcholinesterase reaction disappeared completely after bilateral removal of the superior cervical ganglion, an observation which strongly suggests that the acetylcholinesterase fibres of the pineal are sympathetic axons originating from the superior cervical gland. There are intensely acetylcholinesterase-positive nerve cell bodies in the superior cervical ganglion of the rat, and it is quite possible that the cholinesterase-positive fibres originate from these ganglion cells of the superior cervical ganglion, of which many at the same time exhibit an intense formaldehyde-induced fluorescence (see Eränkö & Härkönen, 1964).

Further light on this problem is shed by the electron microscopic observations of the present study. As has been seen previously in many studies (see, e.g., Arstila & Hopsu, 1964; Bondareff, 1965; Pellegrino De Iraldi *et al.*, 1965; Arstila, 1966), the terminal nerve fibres of the pineal gland regularly contain, after fixation in formaldehyde or glutaraldehyde and further fixation in osmium tetroxide, both empty and small dense-cored synaptic vesicles, in addition to occasional large dense-cored vesicles. The small dense-cored vesicles are believed to be amine-containing ones, and thus indicators of an aminergic nerve fibre (see Grillo, 1966; Eränkö, 1967*a*; Rechardt, 1969). The empty vesicles shown after glutaraldehyde-osmium tetroxide fixation, together with the small dense-cored vesicles in the pineal nerve endings, are probably originally also amine-containing vesicles which have lost their dense core during the less efficiently immobilizing glutaraldehyde fixation. After potassium permanganate fixation a dense core is seen in all vesicles of amine-containing nerve fibre varicosities (see Eränkö, 1967*a*; Hökfelt, 1968). That dense-cored vesicles can be seen after formaldehyde-osmium tetroxide fixation in the pineal gland, but not in most sympathetic nerve fibres elsewhere, can be understood from observations by Jaim-Etcheverry & Zieher (1968) showing that formalin fixation of vesicles containing 5-hydroxytryptamine retains the reactivity towards osmium tetroxide which makes the dense core visible in them, while noradrenaline-containing vesicles show no dense core after formalin treatment, although a dense core can be demonstrated in some of them by the combination of glutaraldehyde and osmium tetroxide fixation.

Electron microscopically, the acetylcholinesterase reaction was observed in the present study beyond any doubt on axons containing small dense-cored vesicles, i.e. amine-containing

nerve fibres. Together with the observations on the similar patterns of the fluorescent and the acetylcholinesterase-positive fibres and on the disappearance of the acetylcholinesterase reaction after sympathectomy, this observation leaves little doubt that the nerve fibres of the pineal which exhibit the acetylcholinesterase reaction are indeed monoaminergic and originate from the sympathetic ganglion.

The authors of the present study cannot between themselves fully agree about the fine localization of the reaction: some of us (L.E., O.E.), who prefer to use a short fixation in formaldehyde, tend to agree with Robinson (1969), who demonstrated an acetylcholinesterase reaction both in the pre-synaptic membrane and the post-synaptic membrane, an empty space between, while others (L.R., A.C.), using glutaraldehyde fixation, tend to believe that the true localization is in the cleft between the two synaptic membranes. However, this divergence of opinions should not detract from the main point of the present study, namely that a definite acetylcholinesterase reaction was observed as a marker of the monoaminergic sympathetic nerve fibres.

The question then arises as to whether the acetylcholinesterase reaction can be used as an indicator of the participation of acetylcholine in the function of the sympathetic fibre, e.g. in the fashion proposed by Burn & Rand (1965). In this connection it is of interest that pharmacological evidence has been obtained of a cholinergic link in the light-stimulated pathway from the eye to the pineal via the superior cervical ganglion (Wartman *et al.*, 1969). However, it seems possible from their data that the link is outside the pineal, e.g. in the pre-ganglionic synapses to the superior cervical ganglion.

Addendum

After the present manuscript was completed, Prof A. B. M. Machado kindly sent us a manuscript (Machado & Lemos, 1970) in which results of their independent study are reported, indicating in agreement with our work acetylcholinesterase activity in nerve fibres of the rat pineal and disappearance of these fibres after bilateral sympathectomy.

References

ARSTILA, A. (1966). Electron microscopic studies on the structure and histochemistry of the pineal gland of the rat. *Neuroendocrin.* Suppl. 2, 1–101.

ARSTILA, A. & HOPSU, V. K. (1964). Studies on the rat pineal gland. I. Ultrastructure. *Annls Acad. Sci. fenn. A.* **113,** 1–21.

ARVY, L. (1961). Contribution à la connaissance de la glande pinéale de Bos taurus L., d'Ovis aries L. et de Sus scrofa L. *C. r. Séance Acad. Sci. (Paris)* **253,** ii, 1361–3.

BONDAREFF, W. (1965). Submicroscopic morphology of granular vesicles in sympathetic nerves of rat pineal body. *Z. Zellforsch. mikrosk. Anat.* **67,** 211–18.

BURN, J. H. & RAND, M. J. (1965). Acetylcholine in adrenergic transmission. *A. Rev. Pharmac.* **5,** 163–82.

ERÄNKÖ, O. (1967*a*). Histochemistry of nervous tissues: catecholamines and cholinesterases. *A. Rev. Pharmac.* **7,** 203–22.

ERÄNKÖ, O. (1967*b*). The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *Jl R. microsc. Soc.* **87,** 259–76.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1964). Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. *Acta physiol. scand.* **61,** 299–300.

ERÄNKÖ, O., HÄRKÖNEN, M., KOKKO, A. & RÄISÄNEN, L. (1964). Histochemical and starch-gel electrophoretic characterization of desmo- and lyo-esterases in the sympathetic and spinal ganglia of the rat. *J. Histochem. Cytochem.* **12,** 570–81.

ERÄNKÖ, O., KOELLE, G. B. & RÄISÄNEN, L. (1967). A thiocholine-lead ferrocyanide method for acetylcholinesterase. *J. Histochem. Cytochem.* **15,** 674–9.

ERÄNKÖ, O. & RÄISÄNEN, L. (1965). Fibres containing both noradrenaline and acetylcholinesterase in the nerve net of the rat iris. *Acta physiol. scand.* **63,** 505–6.

ERÄNKÖ, O. & TERÄVÄINEN, H. (1967). Cholinesterases and eserine-resistant carboxylic esterases in degenerating and regenerating motor end plates of the rat. *J. Neurochem.* **14,** 947–54.

GOMORI, G. (1952). Enzymes. In *Microscopic Histochemistry. Principles and Practise*, pp. 137–221. Chicago: University of Chicago Press.

GRILLO, M. A. (1966). Electron microscopy of sympathetic tissues. *Pharmac. Rev.* **18,** 387–99.

HÖKFELT, T. (1968). *In vitro* studies on central and peripheral monoamine neurons at the ultrastructural level. *Z. Zellforsch mikrosk. Anat.* **91,** 1–74.

HOLMSTEDT, B., LUNDGREN, G. & SJÖQVIST, F. (1962). Determination of acetylcholinesterase activity in normal and denervated sympathetic ganglia of the cat. A biochemical and histochemical comparison. *Acta physiol. scand.* **57,** 187–298.

JAIM-ETCHEVERRY, G. & ZIEHER, L. M. (1968). Cytochemistry of 5-hydroxytryptamine at the electron microscope level. *Z. Zellforsch mikrosk. Anat.* **86,** 393–400.

KAPPERS, J. (1960). The development, topographical relations and innervation of the epiphysis cerebri in the albino rat. *Z. Zellforsch. mikrosk. Anat.* **52,** 163–215.

KAPPERS, J. (1965). Survey of the innervation of the epiphysis cerebri and the accessory pineal organs of vertebrates. *Prog. Brain Res.* **10,** 87–153.

KARNOVSKY, M. J. & ROOTS, L. (1964). A 'direct-coloring' thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12,** 219–21.

KOELLE, G. B. (1951). The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distribution. *J. Pharmac. exp. Ther.* **103,** 153–71.

KOKKO, A., MAUTNER, H. G. & BARRNETT, R. J. (1969). Fine structural localization of acetylcholinesterase using acetyl-β-methylthiocholine and acetylselenocholine as substrates. *J. Histochem. Cytochem.* **17,** 625–40.

LA BELLA, F. S. & SHIN, S. (1968). Estimation of cholinesterase and choline acetyltransferase in bovine anterior pituitary, posterior pituitary, and pineal body. *J. Neurochem.* **15,** 335–42.

MACHADO, A. B. M. & LEMOS, V. P. J. (1970). Histochemical evidence for a cholinergic sympathetic innervation in the rat pineal body. *J. Neuro-visceral Relations* (in press).

OWMAN, C. (1964). New aspects of the mammalian pineal gland. *Acta physiol. scand.* **63,** Suppl. 240, 1–40.

PELLEGRINO DE IRALDI, A., ZIEHER, L. M. & DE ROBERTIS, E. (1965). Ultrastructure and pharmacological studies of nerve endings in the pineal organ; in J. Ariëns Kappers & J. P. Schade's 'Structure and Function of the Epiphysis Cerebri'. *Prog. Brain Res.* **10,** 389–422.

RECHARDT, L. (1969). Electron microscopic and histochemical observations on the supraoptic nucleus of normal and dehydrated rats. *Acta physiol. scand.* **77,** Suppl. 329, 1–79.

ROBINSON, P. M. (1969). A cholinergic component in the innervation of the longitudinal smooth muscle of the guinea-pig vas deferens. *J. Cell Biol.* **41,** 462–76.

THIÉBLOT, L., BASTIDE, P., BLAISE, S., BOYER, J. & DASTUGUE, G. (1966). De l'équipement enzymatique de la glande pinéale. Étude critique et expérimentale. *Ann. Endocrl* **27,** 1–20.

WARTMAN, S. A., BRANCH, B. J., GEORGE, R. & NEWMAN TAYLOR, A. (1969). Evidence for a cholinergic influence on pineal hydroxyindole o-methyl-transferase activity with changes in environmental lighting. *Life Sci.* **8,** 123–70.

Reprinted from
O. Eränkö
PROGRESS IN BRAIN RESEARCH - VOL. 34
Histochemistry of Nervous Transmission
Elsevier Publishing Company, Amsterdam, 1971
Printed in The Netherlands

Small, Intensely Fluorescent Granule-containing Cells in the Sympathetic Ganglion of the Rat

OLAVI ERÄNKÖ AND LIISA ERÄNKÖ

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki (Finland)

In an early study on histochemical demonstration of catecholamines in the sympathetic superior cervical ganglion of the rat (Eränkö and Härkönen, 1963), a diffuse formaldehyde-induced fluorescence due to noradrenaline was reported in all ganglion cells, as well as brilliantly fluorescent small granules in the cytoplasm of many cells and in fibres between the cells. However, amongst typical ganglion cells, occasional small cells were observed to exhibit an extremely bright yellow fluorescence. It was later shown (Eränkö and Härkönen, 1965a) that the fluorescence of these cells, called 'small intensely fluorescent cells' (SIF cells) remained essentially unchanged after division of pre- or postganglionic nerves close to the ganglion, although the latter caused an almost complete disappearance of histochemically demonstrable catecholamines from the cytoplasm of the ordinary ganglion cells.

Since noradrenaline-containing chromaffin cells of the adrenal medulla were known to exhibit an intense formaldehyde-induced fluorescence (Eränkö, 1955b) and since chromaffin cell clusters had been reported in the mesenteric ganglion of the dog (Muscholl and Vogt, 1964), the possibility appeared likely that the SIF cells were, in fact, chromaffin cells. Therefore, in a further study (Eränkö and Härkönen 1965b) complete series of sections were prepared from ganglia fixed in 3.5% potassium dichromate. No or few chromaffin cells were found in the superior cervical ganglion, although numerous small cells were detected in it with formaldehyde-induced fluorescence. However, in the same paper it was also stated that 'cells of the same size and shape as the strongly fluorescent ones were electron microscopically observed to closely resemble the chromaffin cells of the adrenal medulla, containing, like these, numerous intensely osmiophilic granules'. The small cells were therefore considered a new variety of non-chromaffin amine-storing cells, and because the colour of the fluorescence was yellow, 'a monoamine, perhaps 5-hydroxytryptamine', was thought to be stored in the secretory granules of the SIF cells in the same manner as catecholamines in the adrenal medulla.

Soon after our first report on the SIF cells in the superior cervical ganglion of the rat, the presence of SIF cells was confirmed not only in the superior cervical ganglion of the rat but in other sympathetic ganglia and other species as well (Norberg and Hamberger, 1964; Owman and Sjöstrand, 1965; Norberg and Sjöqvist, 1966; Csillik, Kalman and Knyihar, 1967; Jacobowitz, 1967; Olson, 1967). Subsequent

References pp. 50–51

electron microscopic studies also confirmed the presence of granules resembling those in the chromaffin cells in small cells of a shape similar to the SIF cells, and there is little doubt at present that these granular cells are indeed identical with the SIF cells (Grillo, 1966; Williams, 1967; Elfvin, 1968; Siegrist *et al.*, 1968; Hökfelt, 1969; Matthews and Raisman, 1969; Williams and Palay, 1969; Watanabe, 1970). Electron microscopic evidence has further indicated that these small cells receive afferent, apparently non-adrenergic synapses from preganglionic fibres and send efferent synapses to dendrites of sympathetic ganglion cells (Siegrist *et al.*, 1968; Matthews and Raisman, 1969; Williams and Palay, 1969). Close contacts with blood vessels have also been reported (Siegrist *et al.*, 1968; Matthews and Raisman, 1969).

Data on the nature of the monoamines contained in the SIF cells are somewhat confusing. Muscholl and Vogt (1964) found adrenaline and noradrenaline in the chromaffin-cell-containing inferior mesenteric ganglion of the dog, and Owman and Sjöstrand (1965) found a correlation between the occurrence of small cells and adrenaline in the pelvic ganglia of some species. Our early, expressly tentative guess of 5-hydroxytryptamine in the superior cervical ganglion (Eränkö and Härkönen, 1965b) was rejected by Norberg, Ritzén and Ungerstedt (1966), who reported that dopamine or noradrenaline is responsible, as judged by microspectrofluorimetry. Recently, Björklund *et al.*, (1970), who used hydrochloric acid vapour to differentiate between dopamine and noradrenaline (Björklund *et al.*, 1968), reported that the amine of the SIF cells of the sympathetic ganglia of the pig, the cat and the rat is dopamine.

Few reports are available on the development, number and distribution of the SIF cells. Norberg and Sjöqvist (1966) considered that the 'small number and scattered distribution of these cells would indicate that they are of no major physiological importance' and Norberg *et al.* (1966) counted 568 SIF cells in 39 sections of 8 serially sectioned superior cervical ganglia of the rat, while Matthews and Raisman (1969) found in a single superior cervical ganglion of the rat 30 clusters of small cells, including 6 larger groups.

The present study was undertaken to study the constancy, number and distribution of the SIF cells, as yet almost unexamined, as well as the problem of the amine responsible for their intense formaldehyde-induced fluorescence.

MATERIAL AND METHODS

Rats of the Sprague–Dawley strain were briefly anaesthetized with ether and killed by cutting the vertebral column and the aorta with scissors. The superior cervical ganglia were removed and spread on an aluminium foil marked to show the distal and proximal end of the ganglion. The ganglia were frozen by immersion in isopropane precooled with liquid nitrogen and dried under vacuum at —50°C. After complete drying the temperature was increased gradually to about plus 40°C, the vacuum was broken and the ganglia transferred to a Petri dish for exposure to formaldehyde vapour. This was generated from paraformaldehyde powder equilibrated with 60% relative humidity, using different temperatures of exposure. For details of the technique see Eränkö (1967).

For serial sectioning, the ganglia exposed to formaldehyde vapour were directly transferred in the following mixture of epoxy resin: Araldite 502, 15 ml; Epon 812, 25 ml; dibutyl phthalate, 4 ml. To 1.8 ml of this mixture was added 2.2 ml of dodecinyl succinic anhydride and 8 drops of benzyl dimethylamine. They were then poured into flat dishes made of aluminium foil, and the resin was hardened by incubation at 45°C overnight. Serial sections were cut at 5 μm with the LKB pyramitome, using a glass knife. The sections were transferred dry onto slides, 24 sections on each slide, and mounted in Entellan (E. Merck). All sections were examined in a fluorescence microscope using a 3-mm BG 3 and a TAL 403 nm interference filter by Schott for excitation and Leitz K 470 filter for emission. The number of SIF cells was counted in each section, denoting their location in the ganglion and the number of cells in each group. The error due to section thickness was compensated for by using the Floderus formula (see Eränkö, 1955a).

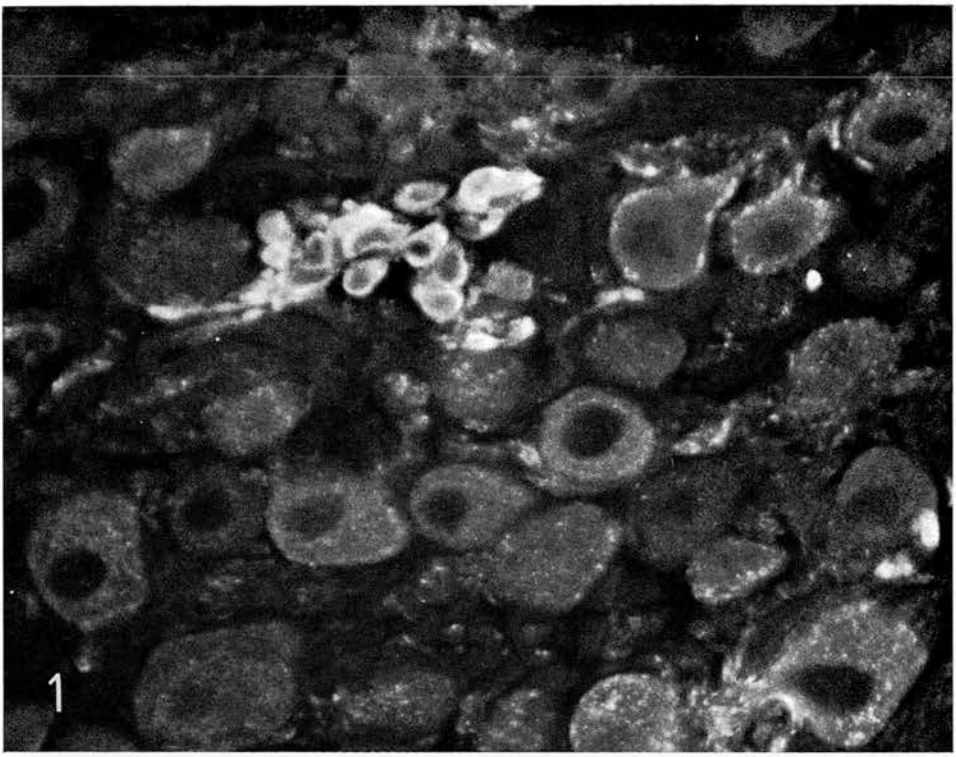
For microspectrofluorimetric analysis, freeze-dried ganglia exposed to formaldehyde vapour were quickly embedded in paraffin wax through xylene. Sections cut at 5 μm were transferred dry onto slides. The paraffin wax was removed with toluene and the sections were mounted in paraffin oil. Excitation and emission spectra were recorded before and after exposure for 5 seconds – 30 minutes to hydrochloric acid vapour generated at room temperature from concentrated hydrochloric acid, density 1.19 g/ml, following the spectral changes in the same cell groups between exposures.

The microspectrofluorimeter is described elsewhere (Eränkö, 1971). It features two Farrand grating monochromators for excitation and emission, respectively, in front of and behind a Zeiss Jena microscope fitted with a Reichert dichroic mirror illuminator to reflect the exciting ultraviolet light on the specimen through the objective yet allowing most of the fluorescent light to pass through to the ocular. A Zeiss 40 × aprochromatic objective was used. A battery-fed RCA 1P28 photomultiplier tube was connected with the y axis of a Moseley $x-y$ recorder, the x axis being run by the wavelength potentiometer.

RESULTS

A typical cluster of SIF cells amongst the larger, less intensely fluorescent, 'ordinary' sympathetic ganglion cells is illustrated in Fig. 1. As in this cell group, the SIF cell profiles were often elongated and sent thin intensely fluorescent processes some distance away from the cell body. Processes originating from individual SIF cells were close to each other and often reached the surface of ordinary sympathetic ganglion cells. Sometimes the SIF cell processes surrounded ganglion cells in intimate contact with them, and their beadings were impinging on the ganglion cell cytoplasm (Fig. 2). Clusters of SIF cells were also observed amongst nerve tracts inside the ganglion (Fig. 3), in preganglionic or postganglionic nerve trunks near the ganglion (Fig. 4), and sometimes directly on ganglion cell bodies (Fig. 7).

The SIF cells were very often grouped around small blood vessels (Figs. 5–8), and if a blood vessel was not visible near or within a group of SIF cells in a section, it was usually possible to find such a vessel in nearby sections cut through the same



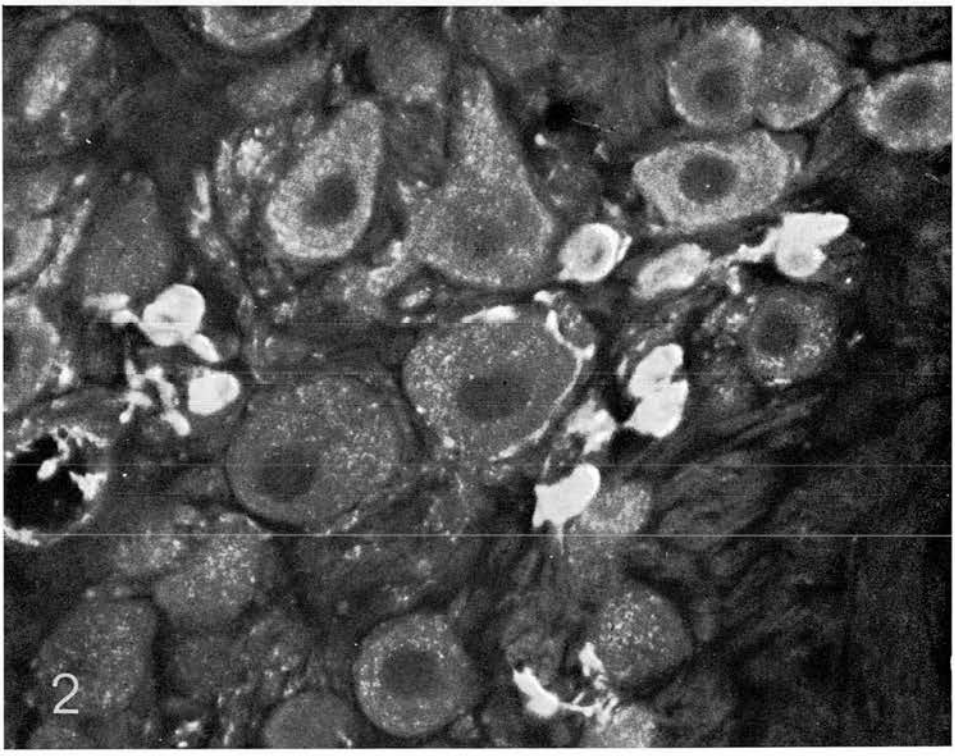


Fig. 1. A group of SIF cells surrounded by typical, less intensely fluorescent sympathetic ganglion cells in the superior cervical ganglion of an adult rat. Magnification 470 ×.

Fig. 2. SIF cells amongst ordinary ganglion cells. The ganglion cell in the centre is surrounded by a slender SIF cell process with beadings. Magnification 470 ×.

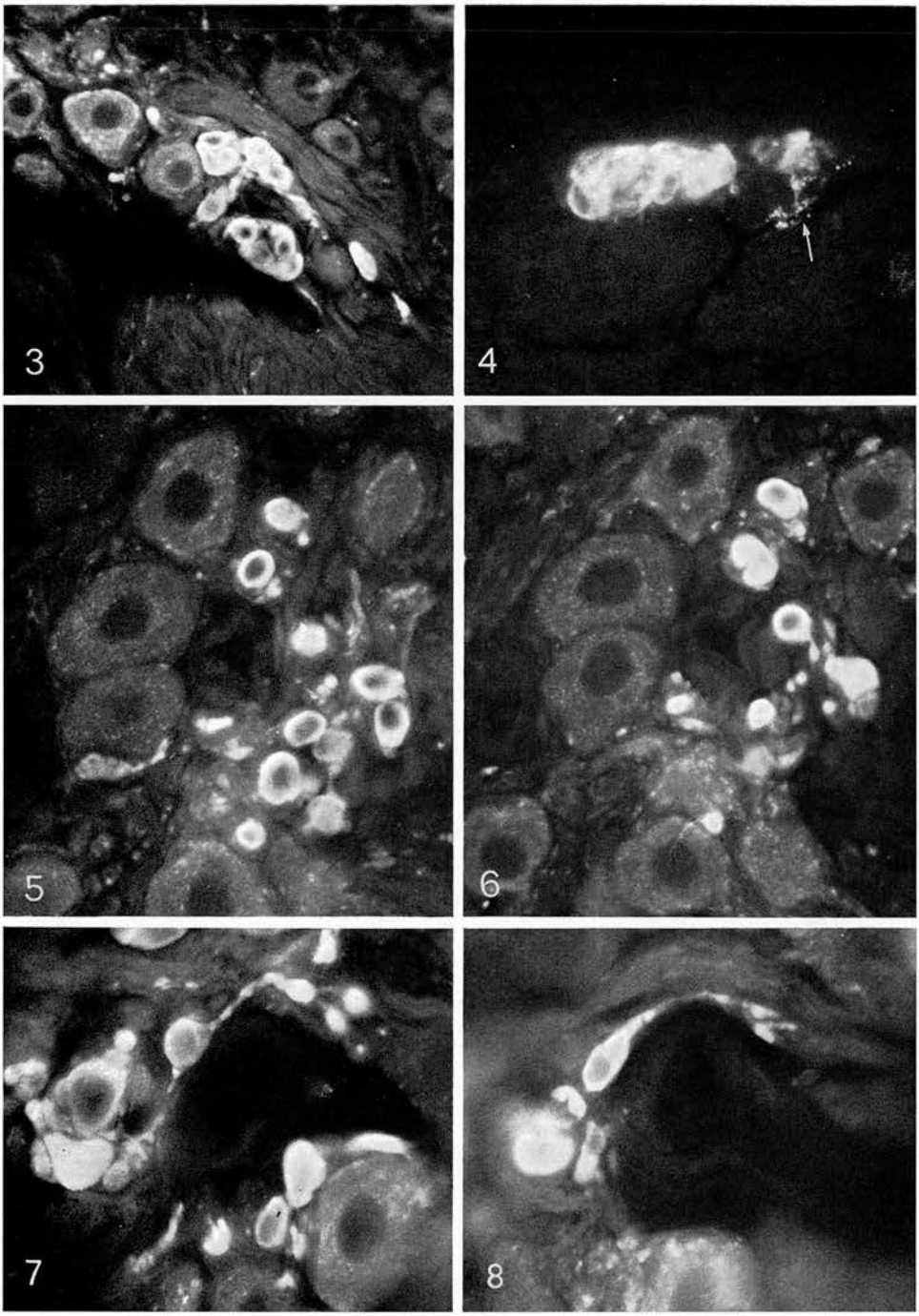


Fig. 3. Blood vessel surrounded by a cluster of SIF cells amongst nerve tracts. 310 ×.
Fig. 4. Typical, dense SIF cell cluster in a postganglionic nerve trunk. Arrow indicates a mast cell. 310 ×.
Fig. 5. Blood vessel surrounded by SIF cells and their processes. On the left a SIF cell in close contact with a ganglion cell. 490 ×.
Fig. 6. Neighbouring section to that shown in Fig. 5. Processes originating from cell bodies in Fig. 5 are seen. 490 ×.
Fig. 7. A SIF cell riding on a blood vessel and sending beaded processes to two directions. On lower right, SIF cells on a ganglion cell. 490 ×.
Fig. 8. Neighbouring section to that shown in Fig. 7. Another SIF cell riding on the same vessel sends a branching process in the nearby neuropil. 490 ×.

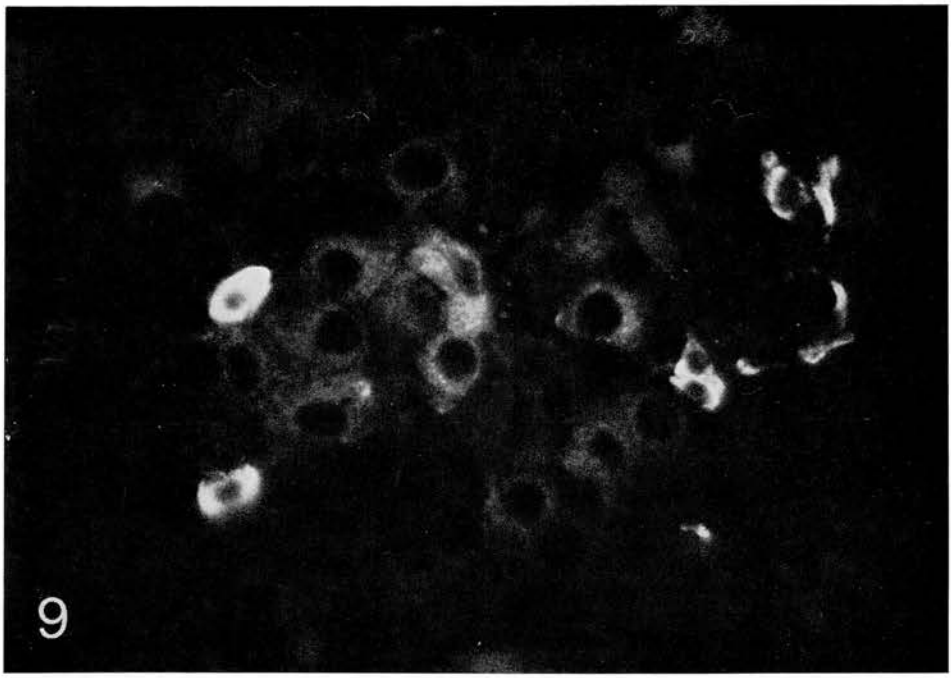


Fig. 9. Superior cervical ganglion of a newborn rat. The ganglion cells are still small and show a weak, even, cytoplasmic fluorescence. On the left, two solitary SIF cells can be seen. On the right, a loose cluster of SIF cells. 490 ×.

cluster of SIF cells. The processes of the SIF cells were often in close proximity to the vessel walls, and sometimes the SIF cells themselves were on a blood vessel (Figs. 7 and 8).

In the sympathetic ganglia of newborn rats and those a few days old, the ordinary ganglion cells were smaller and showed a diffuse cytoplasmic fluorescence. The SIF cells observed in each section were often solitary (Fig. 9, left), in contrast to the SIF cells of adult rats, which formed groups as a rule. As can be seen on the right in Fig. 9, groups of SIF cells were also to be found in young ganglia, although the cells were not usually as close to each other as in the adult ganglia.

The grouping and distribution of the SIF cells in the superior cervical ganglion is schematically illustrated in Fig. 10 in which the number of SIF cells in each cluster is given by the figure nearby. Table 1 gives the number of grouped and individual SIF cells in the left superior cervical ganglion of 5 adult male rats and 3 young rats. The number of individual SIF cells, of SIF cell groups and of grouped SIF cells was reasonably constant in adult animals. In the young ganglia, the total number of SIF cells was almost the same as that in adult ganglia, while only a small part of these cells were observed to form groups.

Emission and excitation spectra of ordinary ganglion cells and SIF cells are compared in Fig. 11 with similar spectra obtained from noradrenaline- and dopamine-incubated iris used as references (Eränkö and Eränkö, 1971). It is obvious that all

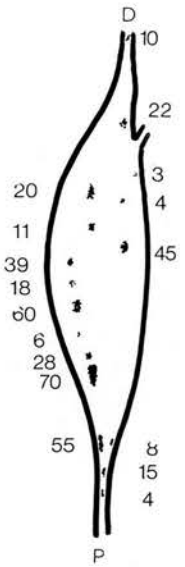


Fig. 10. Drawing illustrating the grouping of SIF cells in an adult superior cervical ganglion. The number of cells in each group is indicated by the figure beside it. D, distal, P, proximal end of the ganglion.

TABLE I

NUMBER OF SIF CELL GROUPS AND SIF CELLS IN THE SUPERIOR CERVICAL GANGLION OF YOUNG AND ADULT RATS

Age (days)	*Cell groups in the ganglion*						*Solitary cells*	*Total cells*
	Distal		*Central*		*Proximal*			
	Groups	*Cells*	*Groups*	*Cells*	*Groups*	*Cells*		
Newborn	0	0	5	160	1	13	199	372
1	1	18	6	180	2	61	174	433
3	0	0	5	222	0	0	326	548
90	9	281	10	231	2	51	15	578
90	12	283	6	170	1	40	10	503
90	14	566	2	19	8	195	14	794
90	5	62	5	86	13	262	19	429
90	8	338	7	192	14	442	14	986

spectra are essentially similar, indicating that catecholamines are responsible for the formaldehyde-induced fluorescence of the SIF cells. It should be noted that 5-hydroxytryptamine produced an entirely different spectrum (not illustrated here), in accordance with previous observations, by Corrodi and Hillarp (1964).

The effect of hydrochloric acid vapour on the excitation spectra of the fluorescent compounds formed from noradrenaline and dopamine is shown in Fig. 12, which

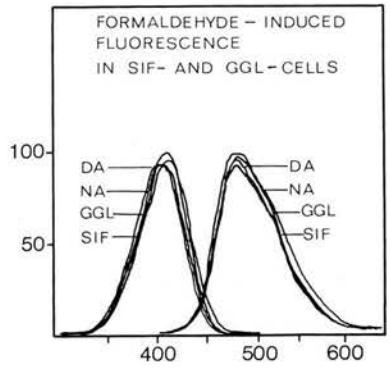


Fig. 11. Excitation (left) and emission (right) spectra of the formaldehyde-induced fluorescence in ganglion (GGL) and SIF cells, as well as in nerve fibres of iris containing noradrenaline (NA) or dopamine (DA).

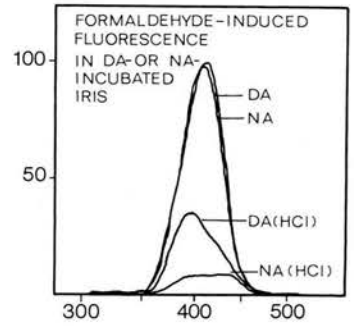


Fig. 12. Excitation spectra of formaldehyde-induced fluorescence in nerve fibres of iris containing noradrenaline (NA) or dopamine (DA) before and after exposure to hydrochloric acid (HCl) vapour

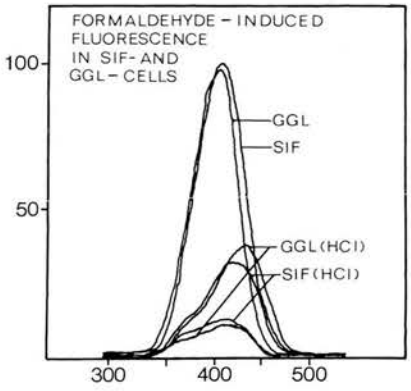


Fig. 13. Excitation spectra of formaldehyde-induced fluorescence in ganglion (GGL) and SIF cells before (high curves) and after exposure for 30 sec (middle curves) or 2 min (lowest curves) to hydrochloric acid (HCl) vapour. The spectra obtained after HCl treatment are in both cases similar to those obtained with noradrenaline.

illustrates the typical difference between these two amines: hydrochloric acid caused a rapid weakening of the noradrenaline fluorophore, while the intensity of the dopamine fluorophore was less affected, the excitation maximum shifting towards shorter wavelengths. The effect of hydrochloric acid on the excitation spectra of the formaldehyde-induced fluorescence of the SIF cells and ordinary ganglion cells is shown in Fig. 13. It is obvious that the spectra are closely similar for both cell types before and after exposure to hydrochloric acid. Since the spectrum continually changed upon exposure, two sets of spectra are shown, after 30 seconds' and 2 minutes' exposure to hydrochloric acid of the same cell groups. Comparison with Fig. 12 shows that the spectra obtained after the 2-minute exposure to hydrochloric acid from the ganglion cells and from the SIF cells were both similar to the spectrum of similarly treated noradrenaline in Fig. 12.

It is important to note in this connection that exposure to hydrochloric acid causes a sequence of changes in the excitation spectra of both noradrenaline and dopamine: first a shift of the excitation maximum to the left and a decrease in the fluorescence intensity, then a shift in the excitation maximum back towards the longer wavelengths and a new increase in the fluorescence intensity (Eränkö and Eränkö, 1970). However, these changes occur more rapidly with noradrenaline than with dopamine, thus allowing discrimination between these amines, but only if other factors, which also affect the rapidity of these changes, are kept constant. Therefore, it is essential to follow, in the same structure, changes induced by consecutive short exposures to hydrochloric acid in the excitation spectra. In the present case this was easy because the SIF cells and the ganglion cells were in the same section and they were thus necessarily exposed to hydrochloric acid and otherwise treated in an identical way. The spectra of the SIF cells treated with hydrochloric acid after formaldehyde clearly showed that the amine they contained was not dopamine.

Since the fluorescence intensity of the SIF cells reached its normal level after just a 10-min exposure to formaldehyde vapour from paraformaldehyde powder equilibrated with 60% relative humidity at 50°C, it seems apparent that noradrenaline is responsible instead of adrenaline, which reacts more slowly (see Eränkö, 1964, 1967).

DISCUSSION

Microspectrofluorimetric observations of the present study clearly indicated that the SIF cells of the superior cervical ganglion of our rats contained noradrenaline rather than dopamine. This observation is at variance with the only previous study in which dopamine and noradrenaline have been differentiated microspectrofluorimetrically (Björklund *et al.*, 1970). Methodological differences are not likely to be responsible because the typically different spectral changes due to hydrochloric acid in noradrenaline and dopamine fluorophores were clearly enough observed in both studies (Björklund *et al.*, 1970; Eränkö and Eränkö, 1971). The main part of the study by Björklund *et al.* (1970) was apparently done with the pig and the cat, with only a passing note on the rat. The presence of dopamine in the SIF cells of one species does not rule out the presence of noradrenaline or even adrenaline (*cf.* Muscholl and

References pp. 50–51

Vogt, 1964; Owman and Sjöstrand, 1965) in the SIF cells of another species, or perhaps even of a different strain of the same species. Moreover, it is not impossible that there might be different types of SIF cells with different amines in different ganglia of the same individual animal, just as there are different types of mast cells containing either dopamine, 5-hydroxytryptamine or histamine (Adams-Ray *et al.*, 1964; Eränkö and Kauko, 1965), or that functional changes may interfere with the beta-hydroxylation or *N*-methylation mechanisms of these cells, resulting in failure of dopamine to be converted into noardrenaline or adrenaline. It would not be surprising to find even 5-hydroxytryptamine in some SIF cells, even if our early, expressly tentative, suggestion that the rat SIF cells might perhaps contain 5-hydroxytryptamine (Eränkö and Härkönen, 1965b) did not prove to be correct. Such a possibility is obvious in view of the observation that not only mast cells but also the sympathetic fibres of the pineal gland originating from the superior cervical ganglion contain appreciable concentrations of 5-hydroxytryptamine (Owman, 1964).

Our earlier observation (Eränkö and Härkönen, 1965b) that the chromaffin reaction is negative in the SIF cells of the superior cervical ganglion of the rat has been confirmed (Norberg *et al.*, 1966). The negative chromaffin reaction is at first sight somewhat surprising considering that noradrenaline was demonstrated in these cells, an amine which readily gives the chromaffin reaction in the adrenal medulla (*e.g.* Coupland, 1965). However, the discrepancy is probably more apparent than real. For the chromaffin reaction to be positive it is necessary that the reactive amine remain *in situ* until dichromate dissolved in water has formed a precipitate with it. The reaction is therefore positive only in those cells in which the amine is relatively firmly bound, such as is the case in the adrenal medulla and in the enterochromaffin cells, while noradrenaline-containing structures with smaller storage granules, such as the sympathetic nerve terminals, do not exhibit a positive chromaffin reaction, even if the local concentration of noradrenaline in itself should be sufficient for the reaction. The explanation lies in the fine structural differences of the granular vesicles in which the amines are bound.

In agreement with the observation from our laboratory that the SIF cells contain granules resembling those in the adrenal medullary cells (Eränkö and Härkönen, 1965b), several authors have observed typical granular vesicles in the SIF cells (Grillo, 1966; Williams, 1967; Elfvin, 1968; Siegrist *et al.*, 1968; Hökfelt, 1969; Matthews and Raisman, 1969; Williams and Palay, 1969; Watanabe, 1970). However, as was pointed out by Matthews and Raisman (1969), the granular vesicles of the small cells of the superior cervical ganglion of the rat are much smaller, about 100 nm, than those of the adrenal medullary cells but much larger than the granular vesicles in ordinary sympathetic ganglion cells. Thus, the SIF cells are in this respect intermediate between sympathetic ganglion cells and adrenal medullary chromaffin cells. Very likely there are differences dependent on the species and on the ganglion concerned in the size of the granular vesicles in the SIF cells. Although no chromaffin cells can be normally found in the rat sympathetic ganglia (Lempinen, 1964; Eränkö and Härkönen, 1965b; Coupland, 1965; Norberg *et al.*, 1966), which contain numerous SIF cells, this does not apply to all species: groups of chromaffin cells have been

reported in sympathetic ganglia of several species (Kohn, 1903; Iwanow, 1932; Muscholl and Vogt, 1964). However, loose usage of the term 'chromaffin' can be confusing. While Siegrist *et al.* (1968) felt that 'with the electron microscope there can be no doubt about the presence of chromaffin cells' in the superior cervical ganglion of the adult rat, this is not so; and Williams and Palay (1969) rightly maintain that 'the term cannot be used properly on the basis of electron microscopic appearances,' when this reaction is not carried out, or proves negative. It remains to be examined whether the true chromaffin cells observed in the sympathetic ganglia have similar ultrastructural features to the SIF cells of the rat.

In adult rats, the SIF cells were almost always grouped into clusters near blood vessels. The close relation to blood vessels has previously led to the assumption that these cells might serve an endocrine function (Eränkö and Härkönen, 1965b). Such a function would indeed be compatible with the electron microscopic observation that fenestrated capillaries similar to those of the adrenal medulla are often seen close to the small granular cells (Siegrist *et al.*, 1968; Matthews and Raisman, 1969).

On the other hand, the close association of the SIF cell processes with the ganglion cell bodies and the intercellular neuropil strongly suggests interaction of a nervous nature, also in cells located close to blood vessels. Indeed, efferent synapses have been clearly demonstrated electron microscopically in these cells (Siegrist *et al.*, 1968; Matthews and Raisman, 1969; Williams and Palay, 1969). The efferent synaptic contacts from the small granular cells have been reported mainly in contact with the dendrites of sympathetic ganglion cells (Matthews and Raisman, 1969; Williams and Palay, 1969), which fits in well with the present observation of fluorescent SIF cell processes in the intercellular neuropil, in which the dendrites lie. However, it is of interest that the long, slender processes of the SIF cells were in the present study observed frequently to be in contact with the ganglion cell body, a phenomenon not described with the electron microscope.

Since afferent synapses, apparently from preganglionic fibres, have also been observed on the small granular cells (Williams, 1967; Siegrist *et al.*, 1968; Matthews and Raisman, 1969; Williams and Palay, 1969), these cells have been presumed to function as interneurons, presumably mediating intraganglionic inhibitory processes, formerly postulated by Eccles and Libet (1961) without morphological evidence to be due to liberation of adrenaline from intraganglionic chromaffin cells.

An inhibitory effect can be caused (1) if the SIF cells release catecholamine into the blood which subsequently reaches ganglion cells or (2) if the SIF cells send inhibitory impulses through their efferent adrenergic synapses to the ordinary ganglion cells. While the second effect can reach but a limited number of ganglion cells because of relatively short processes of the SIF cells and their cluster formation, a large number of ganglion cells could be affected through the blood circulation (*cf.* adrenal medulla).

The present study showed that the number of SIF cells is reasonably constant in the superior cervical ganglion of the rat, as are grouping of the cells into clusters and distribution of these clusters in the ganglion. Moreover, it was shown that almost as many SIF cells can be seen in the ganglion of newborn rats, although mainly scatter-

References pp. 50–51

ed individually in the ganglion with smaller and looser clusters, as in the adult ganglion. The superior cervical ganglion of a newborn rat is at a very immature stage, and clustering may be an essential feature connected with the SIF cell function which develops later.

Grouping of the SIF cells along blood vessels certainly facilitates their eventual endocrine function. However, light microscopic observations of the present study and electron microscopic description of afferent and efferent synapses, as well as attachment plaques, connecting with other small cells and ganglion cells those small cells which are at the same time in close contact with blood vessels (Matthew and Raisman, 1969), suggests rather that the small organ formed by several SIF cells might perform even more sophisticated functions. Could it be possible that the SIF cell clusters take part in the hormonal regulation of the nervous function of the ganglion by sensing the blood composition? In view of the presence of granular vesicles in the chemoreceptor cells of the carotid body this might be a possibility.

While the grouping of the SIF cells around blood vessels can be due to their endocrine or chemoreceptor function, closeness to blood circulation providing for a suitable hormonal milieu may also be a condition for the formation and survival of these cells. Extra-adrenal chromaffin cells which normally degenerate after birth in the rat, surviving only inside adrenocortical tissue, fail to disappear and hypertrophy if hydrocortisone is administered to the animal in the first few days after birth (Lempinen, 1964). Moreover, adrenaline is already found in these cells during the prenatal period if hydrocortisone is given, although normally only noradrenaline can be found in the extra-adrenal chromaffin tissue of the rat (Eränkö, Lempinen and Räisänen, 1967). Such hormonal effects would perhaps not only explain the perivascular location of the SIF cells but also make possible the later humoral modulation of their nervous and endocrine functions.

ACKNOWLEDGEMENTS

The present work was supported by a personal grant to O. E. from the Sigrid Jusélius Foundation and by a grant to the neurohistochemistry group of the Department of Anatomy from the Medical Research Council of Finland. The skilful technical assistance of Mrs. Tuula Stjernvall and Mr. Jarmo Seppänen is gratefully acknowledged.

REFERENCES

ADAMS-RAY, J., DAHLSTRÖM, A., FUXE, K. AND HILLARP, N.-Å. (1964) *Experientia (Basel)*, **20**, 80.
BJÖRKLUND, A., CEGRELL, L., FALCK, B., RITZÉN, M. AND ROSENGREN, E. (1970) *Acta Physiol. Scand.*, **78**, 334–338.
BJÖRKLUND, A., EHINGER, B. AND FALCK, B. (1968) *J. Histochem. Cytochem.*, **16**, 263–270.
CORRODI, H. AND HILLARP, N.-Å. (1964) *Helv. Chim. Acta*, **47**, 911–918.
COUPLAND, R. E. (1965) *The Natural History of the Chromaffin Cell*, Longmans, London.
CSILLIK, B., KALMAN, G. AND KNYIHAR, E. (1967) *Experientia (Basel)*, **23**, 477–478.
ECCLES, R. M. AND LIBET, B. (1961) *J. Physiol. (Lond.)*, **157**, 484–503.
ELFVIN, L. (1968) *J. Ultrastruct. Res.*, **22**, 37–44.

ERÄNKÖ, O. (1955a) *Quantitative Methods in Histology and Microscopic Histochemistry*. Karger, Basel; Little, Brown & Company, Boston.
ERÄNKÖ, O. (1955b) *Acta Endocrinol.*, **18**, 174–179.
ERÄNKÖ, O. (1964) *J. Histochem. Cytochem.*, **12**, 487–488.
ERÄNKÖ, O. (1967) *J. Roy. microscop. Soc.*, **87**, 259–276.
ERÄNKÖ, O. (1971) (In the course of publication).
ERÄNKÖ, O. AND ERÄNKÖ, L. (1971) *J. Histochem. Cytochem.*, in press.
ERÄNKO, O. AND HÄRKÖNEN, M. (1963) *Acta Physiol. Scand.*, **58**, 285–286.
ERÄNKÖ, O. AND HÄRKÖNEN, M. (1965a) *Acta Physiol. Scand.*, **63**, 411–412.
ERÄNKÖ, O. AND HÄRKÖNEN, M. (1965b) *Acta Physiol. Scand.*, **63**, 511–512.
ERÄNKÖ, O. AND KAUKO, L. (1965) *Acta Physiol. Scand.*, **64**, 283–284.
ERÄNKÖ, O., LEMPINEN, M. AND RÄISÄNEN, L. (1967) *Acta Physiol. Scand.*, **69**, 255–256.
GRILLO, M. A. (1966) *Pharmacol. Rev.*, **18**, 387–399.
HÖKFELT, T. (1969) *Acta Physiol. Scand.*, **76**, 427–440.
IWANOW, G. (1932) *Ergeb. Anat. Entwickl.-Gesch.*, **29**, 87–280.
JACOBOWITZ, D. (1967) *J. Pharmacol. Exp. Therap.*, **158**, 227–240.
KOHN, A. (1903) *Arch. Mikroskop. Anat.*, **62**, 263–365.
LEMPINEN, M. (1964) *Acta Physiol. Scand.*, **66**, suppl. **231**, 1–91.
MATTHEWS, M. R. AND RAISMAN, G. (1969) *J. Anat. (Lond.)*, **105**, 255–282.
MUSCHOLL, E. AND VOGT, M. (1964) *Brit. J. Pharmacol.*, **22**, 193–203.
NORBERG, K.-A. AND HAMBERGER, B. (1964) *Acta Physiol. Scand.*, **63**, suppl. **238**, 1–42.
NORBERG, K.-A., RITZÉN, M. AND UNGERSTEDT, U. (1966) *Acta Physiol. Scand.*, **67**, 260–270.
NORBERG, K.-A. AND SJÖQVIST, F. (1966) *Pharmacol. Rev.*, **18**, 743–751.
OLSON, L. (1967) *Z. Zellforsch.*, **81**, 155–173.
OLSON, L. (1970) *Histochemie*, **22**, 1–7.
OWMAN, C. (1964) *Acta Physiol. Scand.*, **63**, suppl. **240**, 1–40.
OWMAN, C. AND SJÖSTRAND, N. O. (1965) *Z. Zellforsch.*, **66**, 300–320.
SIEGRIST, G., DOLIVO, M., DUNANT, Y., FOROGLOV-KERAMEUS, C., DE RIBAUPIERRE, F. AND ROULLIER, C. (1968) *J. Ultrastruct. Res.*, **25**, 381–407.
WATANABE, H. (1970) *Experientia*, **26**, 69–70.
WILLIAMS, T. H. (1967) *Nature (Lond.)*, **214**, 309–310.
WILLIAMS, T. H. AND PALAY, S. L. (1969) *Brain Res.*, **15**, 17–34.

Histochemical Journal, **3** (1971), 357–363

Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of the pineal body of the rat after chemical sympathectomy with 6-hydroxydopamine

OLAVI ERÄNKÖ *and* LIISA ERÄNKÖ

Department of Anatomy, University of Helsinki, Finland

Received 17 May 1971

Synopsis. Newborn albino rats were injected daily for 8 days with 50 μg/g of 6-hydroxydopamine. They were killed 3 weeks after the last injection together with untreated litter mate controls. Monoamines were demonstrated histochemically in the pineal body, in the iris and in the superior cervical ganglion with the formaldehyde-induced fluorescence method. Acetylcholinesterase was demonstrated in the pineal using acetylcholine as substrate and tetraisopropy-pyrophosphoramide (iso-OMPA) to inhibit non-specific cholinesterases.

Treatment with 6-hydroxydopamine caused a complete disappearance of amine-containing fibres from the pineal, whereas some fluorescent ganglion cells remained in the superior cervical ganglion and in some rats a few amine-containing fibres in the iris. Acetylcholinesterase activity, located in fine nerve fibres of the pineal body, disappeared completely after treatment with 6-hydroxydopamine.

Since 6-hydroxydopamine causes a selective destruction of the aminergic sympathetic fibres, it is concluded that the disappearance of the acetylcholinesterase activity indicates that in the pineal body this enzyme activity is located exclusively in truly aminergic nerve fibres.

Introduction

It has been shown in two independent studies (Eränkö *et al.*, 1970; Machado & Lemos, 1971) that the acetylcholinesterase activity of the fine nerve net of the pineal body of the rat disappears after bilateral removal of the superior cervical ganglion, an operation which also causes disappearance of the formaldehyde-induced amine fluorescence from them (Owman, 1964; Eränkö *et al.*, 1970). It was concluded that the typical aminergic sympathetic axons of the pineal also contain acetylcholinesterase.

It has also recently been shown that a selective destruction of sympathetic nerve endings and ganglia can be achieved by injections of 6-hydroxydopamine (Thoenen & Tranzer, 1968; Angeletti & Levi-Montalcini, 1970). Since the 'chemical sympathectomy' obtained in this way depends on a highly specific uptake of the 'false' transmitter substance, 6-hydroxydopamine,

exclusively by amine-producing cells, it appeared to be of special interest to study its effects on both histochemically demonstrable catecholamines and acetylcholinesterase in the pineal gland. To our knowledge no such study has been carried out as yet. The problem is of special interest in view of the pharmacological evidence recently reviewed by Burn (1971), of a cholinergic link in adrenergic transmission. Catecholamines were also studied in the iris and in the superior cervical ganglion as controls of the effect of 6-hydroxydopamine.

Materials and methods

Experimental

Newborn rats were injected daily for 8 days with 50 μg of 6-hydroxydopamine (F. Hoffmann-LaRoche, Basle) per g body weight, a dosage schedule proposed by Angeletti & Levi-Montalcini (1970). The first injection was given within 12 hr after birth. The animals were killed one month after birth, i.e. about 3 weeks after discontinuation of the 6-hydroxydopamine injections. Newborn rats of each litter were divided into experimental animals to be injected with 6-hydroxydopamine and controls which were untreated. At the time of killing there was no discernible difference in the size, outlook or behaviour of the experimental and control rats of each litter. Sixteen experimental and sixteen control rats were used.

The animals were killed by first giving a light ether anaesthesia and then cutting the back with scissors in the region of the heart. The pineal and both superior cervical ganglia were removed immediately thereafter. They were then either freeze-dried for the demonstration of catecholamines or fixed by immersion in a formaldehyde fixative for the demonstration of acetylcholinesterase activity. The iris was stretched on a slide and allowed to dry at room temperature in a desiccator.

Demonstration of monoamines

Formaldehyde-induced fluorescence (see Eränkö, 1967) was used for the demonstration of the monoamines (noradrenaline and 5-hydroxytryptamine). The pineal bodies and the ganglia were frozen stretched on an aluminium foil by immersion in propane cooled to -190° C with liquid nitrogen. They were then freeze-dried for 8 days *in vacuo* at -45° C in an apparatus with a short-path phosphorus pentoxide trap. They were then warmed to 40° C, the vacuum was broken, and the dry organs were transferred to a petri dish containing paraformaldehyde powder equilibrated with an atmosphere of 60% relative humidity. Formaldehyde gas was generated at 50° C for 30 min and thereafter at 80° C for 1 hr. The iris stretch preparations on the slide were treated similarly.

The pineal bodies and the ganglia were then embedded in an Epon-Araldite mixture (Eränkö & Eränkö, 1971), and 5 μm thick sections were cut dry with a glass knife. The sections and the iris preparations were mounted in Entellan (E. Merck) and examined with a Leitz Ortholux fluorescence microscope fitted with an HBO 200 mercury lamp, two 3 mm thick BG 38 heat-absorbing filters, one 3 mm thick BG 3 filter, a TAL 405 interference filter (all filters by Schott & Gen., Mainz), a Ploem (1971) epi-illuminator and a Leitz ultraviolet absorbing filter K 470.

Demonstration of acetylcholinesterase activity

The pineal bodies were fixed overnight by immersion in ice-cold 3.5% solution of formaldehyde made from paraformaldehyde powder in 0.1 M phosphate buffer, pH 7.4. They were then

rinsed overnight in ice-cold 0.1 M phosphate buffer, pH 7.4, containing 0.3 M sucrose. Frozen sections cut at 10 μm were incubated in the acetylthiocholine mixture modified by Gomori (1952) from Koelle's (1951) original prescription, into which was further added tetraisopropy-pyrophosphoramide (iso-OMPA; L. Light, Colnbrook) to make a 10^{-5} M solution, known from a previous study (Eränkö *et al.*, 1970) to inhibit completely the non-specific cholinesterase activity of the pineal. The incubation was carried out at 37 C overnight, and the copper thiocholine sulphate precipitate was made visible with ammonium sulphide. Before incubation, the sections were pre-incubated in 10^{-5} M iso-OMPA.

Results

Sympathetic ganglia

In agreement with the observations made by Angeletti & Levi-Montalcini (1970), a marked diminution in the size of the superior cervical ganglion was observed in the rats injected with 6-hydroxydopamine. The volume of the ganglia was visually assessed to be about one-tenth of the control ganglia. Fluorescence microscopic examination showed that the remaining small

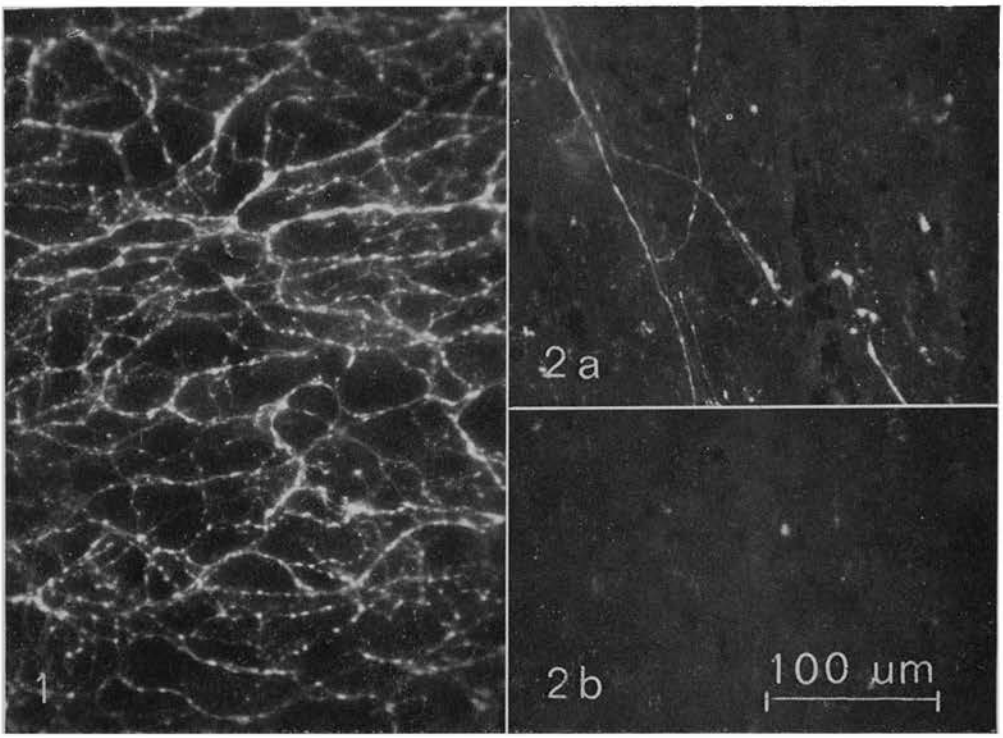


Figure 1. Formaldehyde-induced fluorescence in the noradrenaline-containing nerve net of the rat iris. Typical normal sympathetic nerve net.

Figure 2*a*. Similar fluorescence preparation of the iris of a rat treated with 6-hydroxydopamine. While most fluorescent fibres have disappeared, the few fibres left fluoresce with normal intensity.

Figure 2*b*. Iris of another rat treated with 6-hydroxydopamine. Only weak background fluorescence is visible.

Magnification in all figures is · 225, as indicated by the calibration mark.

ganglion nodules contained nerve cells exhibiting a typical blue-green catecholamine fluorescence in ganglion cells and in small intensely fluorescent cells, also present in the normal sympathetic ganglion (see Eränkö & Eränkö, 1971). A more detailed description of the effects of 6-hydroxydopamine on the ganglion will be reported elsewhere.

Iris

The normal nerve net of the iris was observed in all control animals with its brilliant fluorescence due to noradrenaline (Fig. 1), while the iris preparations made from the rats injected with 6-hydroxydopamine were as a rule entirely devoid of amine fluorescence (Fig. 2*b*). However, in some irises, one or two ramifying axons were seen that exhibited an apparently normal green formaldehyde-induced fluorescence (Fig. 2*a*).

Pineal body

The pineal glands of the rats injected with 6-hydroxydopamine appeared at gross inspection to be of the same size, shape and colour as those of the normal control animals. However, a distinct difference was observed in the formaldehyde-induced fluorescence. In the control pineal bodies, an intense green fluorescence was observed in the sympathetic nerve net of the pineal body, presumably due to both noradrenaline and 5-hydroxytryptamine, while the background of parenchymal cells showed a diffuse yellow 5-hydroxytryptamine fluorescence (Fig. 3); this fluorescence faded rapidly and special pains were taken to preserve it by photographing a fresh field.

In the pineals of the rats injected with 6-hydroxydopamine, on the other hand, the fluorescent nerve net had disappeared altogether, whereas the yellow parenchymal fluorescence was still present, and its intensity was about the same as that in the control parenchyma (Fig. 4).

The distribution of the acetylcholinesterase activity in the pineal bodies of normal rats (Fig. 5) showed a similar pattern as that of the fibres exhibiting formaldehyde-induced fluorescence (Fig. 3). No acetylcholinesterase reaction at all was seen in the pineal of the rats which had been treated with 6-hydroxydopamine (Fig. 6).

Discussion

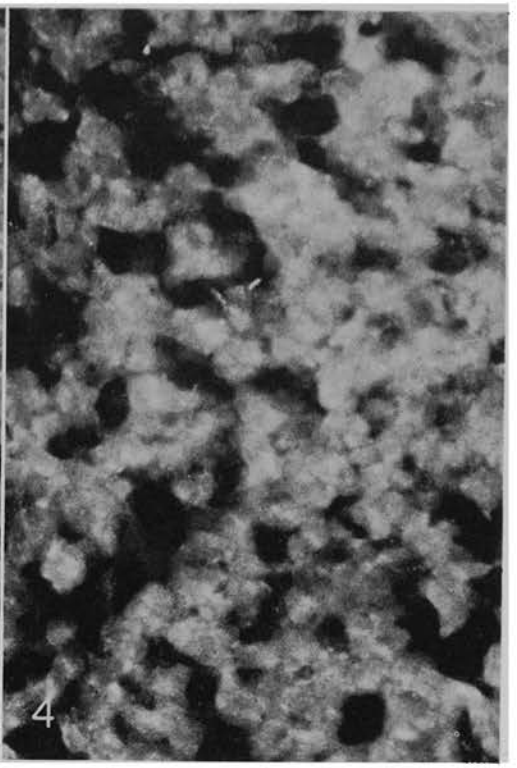
The observations of the present study on the distribution of formaldehyde-induced fluorescence, which was green in the pineal nerve net and yellow in the parenchymal cells, confirms the findings by Owman (1964), who concluded that the nerve fibres contain both their normal transmitter substance, noradrenaline, and 5-hydroxytryptamine taken up from the parenchymal cells, which again contain exclusively the latter amine. The similarity of the nerve fibre pattern demonstrable with the fluorescence method and that made visible by the acetylcho-

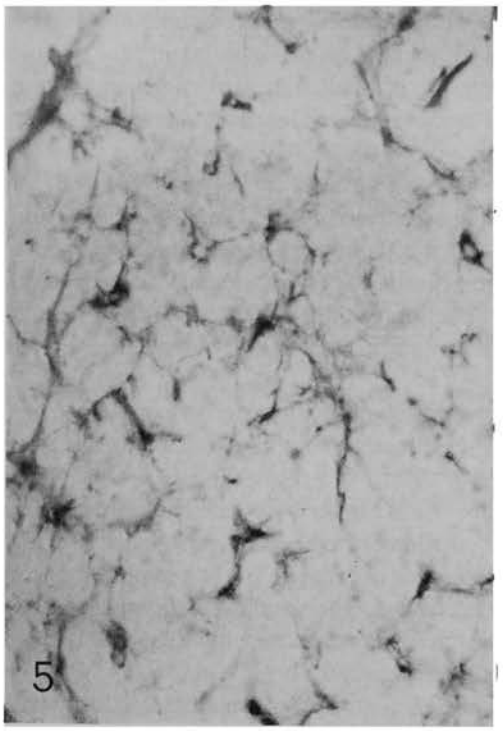
Figure 3. Formaldehyde-induced fluorescence in the pineal body of a control rat. Intensely fluorescent nerve fibres are distinctly visible amongst somewhat less intensely fluorescent parenchymal cells.

Figure 4. Identically handled pineal body from a rat injected with 6-hydroxydopamine. The parenchyma fluorescence is unchanged but the nerve net fluorescence has totally disappeared.

Figure 5. Acetylcholinesterase reaction in the pineal body of a control rat. Nerve fibre pattern resembles that in Fig. 3.

Figure 6. Similar preparation of a rat injected with 6-hydroxydopamine. There is a total loss of acetylcholinesterase activity.





6

linesterase technique, reported in the previous paper from our laboratory (Eränkö *et al.*, 1970), was also confirmed in the present study .

Treatment with 6-hydroxydopamine caused a complete disappearance of fluorescent nerve fibres from the pineal body and an almost complete disappearance of such fibres from the iris. Such disappearance is in conformity with previous chemical and electron microscopic observations by Tranzer & Thoenen (1967, 1968) and Thoenen & Tranzer (1968) in sympathetic nerve terminals. The complete disappearance in the present study of the formaldehyde-induced fluorescence reaction from the pineal body is of interest, because in our material the destruction of the superior cervical ganglion was not quite complete after treatment with 6-hydroxydopamine. Angeletti & Levi-Montalcini (1970), whose treatment schedule with 6-hydroxydopamine we have used in an identical way, reported a complete and permanent destruction. However, it appears from their report that even in their material ganglion cells were still present in the small ganglion remnants of animals treated with 6-hydroxydopamine for three days, although these cells were reported to exhibit abnormal characteristics on electron microscopical examination and to disappear in two weeks after discontinued treatment. Some ganglion cells appear to be more resistant towards 6-hydroxydopamine than others, and it is, therefore, not surprising that some cells survived in our experiments.

From the point of view of the present study, the survival of some ganglion cells and some iris nerve fibres does not matter, because 6-hydroxydopamine caused a complete disappearance of the acetylcholinesterase-positive nerve net from the pineal body. Since the effect of 6-hydroxydopamine is limited exclusively to the catecholamine-containing terminal nerve fibres without affecting at all the nearby cholinergic nerve fibres (Tranzer & Thoenen, 1967; Thoenen & Tranzer, 1968), it is evident that the cholinesterase-positive pineal nerve fibres are truly aminergic sympathetic fibres, whose cholinesterase reaction disappears because 6-hydroxydopamine has destroyed them. This fits in well with the electron microscopic observations that showed that the acetylcholinesterase activity is located on the axon membrane of fibres whose aminergic nature was indicated by their content of small granular vesicles and that the acetylcholinesterase disappeared after bilateral removal of the superior cervical ganglion (Eränkö *et al.*, 1970).

Although a positive acetylcholinesterase reaction, of whose specificity there is hardly any doubt (Eränkö *et al.*, 1964, 1970), can thus be demonstrated in the sympathetic aminergic axons of pineal, it is not clear whether such a reaction is demonstrable in all aminergic fibres. A negative acetylcholinesterase reaction has been reported by Robinson (1969) in adrenergic nerve fibres close to acetylcholinesterase-positive cholinergic fibres. However, further incubation might demonstrate acetylcholinesterase activity even in such fibres, in which the enzyme activity may be less intense.

Even if there appears to be no doubt about the acetylcholinesterase activity of at least some aminergic sympathetic nerve fibres, this does not directly prove that these fibres are also cholinergic and contain acetylcholine (see Eränkö, 1967*a*). To prove *directly* the interesting hypothesis that a single nerve fibre can at the same time use acetylcholine and noradrenaline as transmitter substances (Burn, 1971) would require demonstration of acetylcholine or at least cholinacetylase in the adrenergic nerve fibres. A study is in progress in the present laboratory to find out whether bilateral removal of superior cervical ganglion or chemical sympathectomy with 6-hydroxydopamine affects the choline acetyltransferase activity of the pineal body, in which La Bella & Shin (1968) have demonstrated such activity.

Even if no acetylcholine or choline acetyltransferase activity is ever demonstrated in adrener-

gic nerve centres, the presence of acetylcholinesterase in them is still of great interest. That this enzyme serves a function essential from the point of view of axonal conduction has long been proposed by Nachmansohn (1963).

References

ANGELETTI, P. U. & LEVI-MONTALCINI, R. (1970). Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. Nat. Acad. Sci.* **65,** 114–21.

BURN, H. J. (1971). *The Autonomic Nervous System.* Oxford and Edinburgh: Blackwell Scientific Publications.

ERÄNKÖ, O. (1967*a*). Histochemistry of nervous tissues: catecholamines and cholinesterases. *A. Rev. Pharmacol.* **7,** 202–22.

ERÄNKÖ, O. (1967*b*). The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *Jl R. microsc. Soc.* **87,** 259–76.

ERÄNKÖ, O. & ERÄNKÖ, L. (1971). Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* **34** (in press).

ERÄNKÖ, O., HÄRKÖNEN, M., KOKKO, A. & RÄISÄNEN, L. (1964). Histochemical and starch-gel electrophoretic characterization of desmo- and lyo-esterases in the sympathetic and spinal ganglia of the rat. *J. Histochem. Cytochem.* **12,** 570–81.

ERÄNKÖ, O., RECHARDT, L., ERÄNKÖ, L. & CUNNINGHAM, A. (1970). Light and electron microscopic histochemical observations on cholinesterase-containing sympathetic nerve fibres in the pineal body of the rat. *Histochem. J.* **2,** 479–89.

GOMORI, G. (1952). Enzymes. In *Microscopic Histochemistry. Principles and Practice,* pp. 137–221. Chicago: University of Chicago Press.

KOELLE, G. B. (1951). The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distribution. *J. Pharmac. exp. Ther.* **103,** 153–71.

LA BELLA, F. S. & SHIN, S. (1968). Estimation of cholinesterase and choline acetyltransferase in bovine anterior pituitary, posterior pituitary and pineal body. *J. Neurochem.* **15,** 335–42.

MACHADO, A. B. M. & LEMOS, V. P. J. (1971). Histochemical evidence for a cholinergic sympathetic innervation in the rat pineal body. *J. Neuro-visceral Relations* (in press).

NACHMANSOHN, D. (1963). Actions on axons, and evidence for the role of acetylcholine in axonal conduction. In: *Handbuch der experimentellen Pharmakologie,* pp. 701–40. Ergänzungswerk, Vol. 15 (ed. G. B. Koelle), Springer-Verlag.

OWMAN, C. (1964). New aspects of the mammalian pineal gland. *Acta physiol. scand.* **63,** Suppl. 240, 1–40.

PLOEM, J. S. (1971). The microscopic differentiation of the colour of formaldehyde-induced fluorescence. *Progr. Brain Res.* **34** (in press).

ROBINSON, P. M. (1969). A cholinergic component in the innervation of the longitudinal smooth muscle of the guinea-pig vas deferens. *J. Cell. Biol.* **41,** 462–76.

THOENEN, H. & TRANZER, J. P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **261,** 271–88.

TRANZER, J. P. & THOENEN, H. (1967). Ultramorphologische Veränderungen der sympathischen Nervenendigungen der Katze nach Vorbehandlung mit 5- und 6-Hydroxy-Dopamin. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak,* **257,** 343–4.

TRANZER, J. P. & THOENEN, H. (1968). An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia.* **24,** 155–6.

PRINTED IN GREAT BRITAIN BY THE BROADWATER PRESS LTD, WELWYN GARDEN CITY, HERTFORDSHIRE

Histochemical Journal, **3** (1971), 451–456

Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats

OLAVI ERÄNKÖ *and* LIISA ERÄNKÖ

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received 16 July 1971

Synopsis. Guanethidine is known to cause a loss of catecholamines from sympathetically innervated tissues and sympathetic ganglia in adult animals but its effect on newborn animals has not been examined.

Newborn rats were injected daily with guanethidine (20 mg/kg body weight) for 8 days. They were killed when 1 month-old along with untreated litter mate controls. Catecholamines were demonstrated in the iris, in the pineal body and in sympathetic ganglia, using the formaldehyde-induced fluorescence method.

In the guanethidine-treated rats there was a complete loss of fluorescent nerve fibres from the pineal body and an almost complete loss of similar fibres from the iris. The sympathetic ganglia were reduced to less than 10% of the control ganglia, and the number of nerve cell bodies per unit area was decreased in the ganglion remnants.

It is concluded that guanethidine causes, in newborn rats, an irreversible destruction of most sympathetic neurons, i.e. a chemical sympathectomy closely resembling that obtainable in newborn animals by injections of 6-hydroxydopamine or antiserum to nerve growth factor.

Introduction

Administration of 6-hydroxydopamine to experimental animals leads to selective uptake of this amine in sympathetic nerve terminals and, if the dose is sufficiently high, to destruction of the terminals and loss of intrinsic catecholamines from them (Porter *et al.*, 1963; Stone *et al.*, 1964; Thoenen & Tranzer, 1968). If this drug is administered to newborn animals daily for several days, a permanent destruction follows not only of peripheral sympathetic nerve fibres but of nerve cells in the sympathetic ganglia as well (Angeletti & Levi-Montalcini, 1970*a*; Angeletti, 1971; Eränkö & Eränkö, 1971*a*, *b*). Thus, a 'chemical sympathectomy' is obtained which closely resembles the 'immunosympathectomy' caused in newborn animals by injecting antiserum against the nerve growth factor (Cohen, 1960; Levi-Montalcini & Angeletti, 1966).

While it is known that many catecholamine derivatives and other related compounds are taken up by sympathetic nerve terminals and are even released by a nervous stimulus, serving as false transmitter substances (Thoenen, 1969), no drug other than 6-hydroxydopamine has as yet been shown to cause an irreversible chemical sympathectomy.

Guanethidine has long been known to cause a decrease in the noradrenaline content of sympathetic nerves and ganglion cells (Sanan & Vogt, 1962; Malmfors & Sachs, 1968). It becomes concentrated within the sympathetic neurons by the membrane transport system that pumps noradrenaline into the neuron after its release (Mitchell & Oates, 1970).

To our knowledge the effect of repeated injections of guanethidine on the sympathetic system of newborn animals has not been investigated. Such a study is reported in this paper which presents histochemical evidence of chemical sympathectomy after administration of guanethidine.

Materials and methods

Experimental

Eighteen newborn albino rats were injected with guanethidine (20 mg kg body weight) daily for 8 days, beginning within 12 hr after birth. This is the same administration schedule as that prescribed by Angeletti & Levi-Montalcini (1970*a*) for chemical sympathectomy with 6-hydroxydopamine. Two injected rats died on the fourth week after birth. Litter mates of the injected animals, altogether 16 rats, served as controls. All animals were killed when they were 1 month-old, that is when over 3 weeks had elapsed from the last injection.

Demonstration of catecholamines

For the histochemical demonstration of catecholamines, stretch preparations were made of the iris, while the pineal body, the superior cervical ganglion and the coeliac ganglion were freeze-dried, mounted in a mixture of Epon and Araldite, exposed to formaldehyde vapour and examined by fluorescence microscopy, using techniques whose details have been described in previous papers (Eränkö, 1967; Eränkö & Eränkö, 1971*b*).

Results

General observations

Guanethidine had a long-lasting effect on the growth of the injected animals. Their weight was about 40 g when they were 1 month old while the untreated rats weighed about 60 g. The injected rats were inactive and shaggy.

Iris

The distribution of catecholamines in a 1-month-old control rat is shown in Fig. 1, in which typical fluorescent nerve fibres with their intensely fluorescent synaptic beadings can be seen. Fig. 2 is a similar stretch preparation from a 1-month-old rat which had been injected with guanethidine; no fluorescent nerve fibres can be seen, some cells (macrophages or mast cells?) with fluorescent cytoplasmic granules being the only fluorescent elements in the field.

While such complete loss of catecholamine-containing fibres was observed in the iris of 1 out of the 16 surviving rats treated with guanethidine, a few fluorescent nerve fibres were observed in the iris of 2 injected rats. Even in these 2 rats, well over 90% of the fibres had disappeared but the remaining fibres exhibited a normal fluorescence intensity and the characteristic beadings.

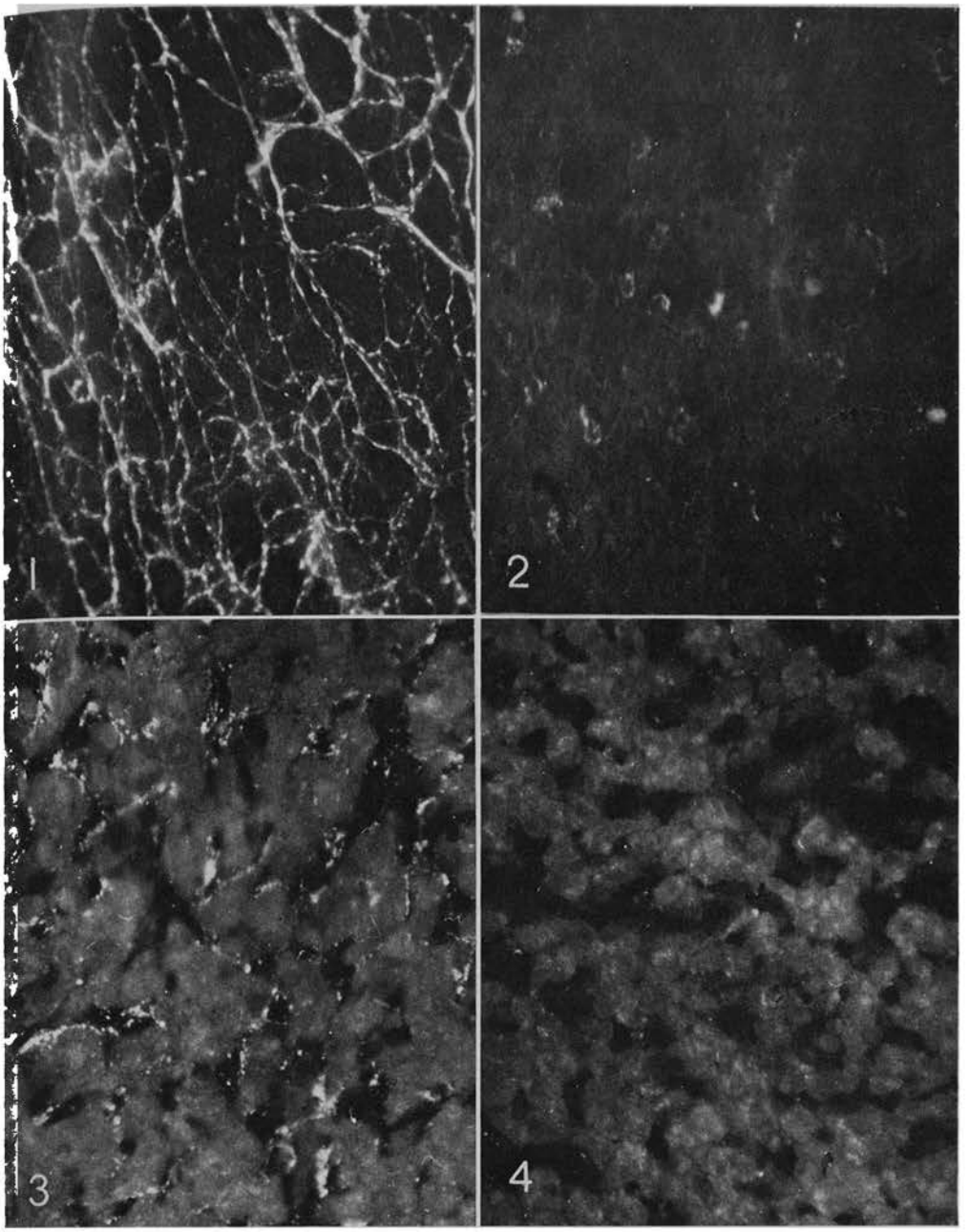


Figure 1. Catecholamine fluorescence (induced by formaldehyde) in the sympathetic nerve net in the iris of a 1-month-old control rat. × 250

Figure 2. The iris of a rat injected with guanethidine daily for 8 days immediately after birth and allowed to recover thereafter for 3 weeks. The fluorescent nerve fibres have disappeared altogether. × 250

Figure 3. Formaldehyde-induced fluorescence of the pineal body of a control rat. Sympathetic nerve fibres exhibit intense fluorescence, the parenchymal cells are moderately fluorescent. × 250

Figure 4. Photograph taken as Fig. 3 from the pineal body of a rat injected during the first 8 days after birth with guanethidine. The parenchymal fluorescence is unchanged but the intensely fluorescent nerve fibres have disappeared. × 250

Pineal body

The fluorescence of the normal pineal body of a control rat is illustrated in Fig. 3, showing th brightly fluorescent sympathetic nerve fibres with still brighter beadings, especially near th vascular spaces visible as irregular black areas in the diffusely fluorescent parenchyma. Sinc Fig. 3 is of a section, the continuity of the nerve fibres is broken, and the network is not visibl in the same way as in the stretched iris (Fig. 1).

All the guanethidine-treated rats, including the 2 rats in whose iris some fluorescent fibre were seen, had totally lost the fluorescent nerve fibres from the pineal, while the parenchyma fluorescence remained unaltered, as can be seen from Fig. 4.

Sympathetic ganglia

Guanethidine administration caused a dramatic diminution of all pre- and paravertebra ganglia in all injected rats. The volumes of the ganglion remnants were assessed visually to b less than 10% of the corresponding volume of the controls. Fluorescence microscopic examination of the superior cervical and coeliac ganglia showed, moreover, that the number of ganglio cells per unit area of section was much decreased in the surviving nodules and nerve fibres too proportionally more space between the ganglion cells than in the control ganglia. The surviving ganglion cells showed an essentially normal cytoplasmic fluorescence. Some of them wer larger than any cells seen in the normal ganglia. Clusters of small, intensely fluorescent cells also present in the normal ganglia, were larger in the ganglia of rats injected with guanethidin than those in the control ganglia.

A more detailed study of the ganglia will be reported elsewhere.

Discussion

Administration of guanethidine to adult animals causes a loss of catecholamines from the sympathetic nerve terminals and the ganglia; this loss is readily reversible (Sanan & Vogt, 1962 Malmfors & Sachs, 1968; Jensen-Holm & Juul, 1970). On the other hand, administration o 6-hydroxydopamine results in real destruction of peripheral sympathetic fibres even in adul animals (Thoenen & Tranzer, 1968), although the sympathetic nerve cell bodies are littl affected (Angeletti & Levi-Montalcini, 1970*a*), and regeneration of the nerve fibres occurs i 2–4 weeks (Thoenen & Tranzer, 1968).

The results of the present study show that administration of guanethidine to newborn rat leads to complete disappearance of the fluorescent nerve fibres from the pineal body and a almost complete loss of such fibres from the iris. Destruction of over 90% of the nerve cell from the sympathetic ganglia caused by the same treatment makes it understandable that n regeneration has occurred in spite of the relatively long recovery time, 3 weeks; regeneratio is not possible because the ganglion cells have been destroyed.

These changes are essentially identical with those obtained after treatment of newborn rat with 6-hydroxydopamine (Angeletti & Levi-Montalcini, 1970*a*; Angeletti, 1971; Eränkö & Eränkö, 1971*a*, *b*). It is interesting that some peripheral fibres and some ganglion cells surviv the guanethidine treatment (the present study), the 6-hydroxydopamine treatment (Angeletti 1971; Eränkö & Eränkö, 1971*a*, *b*) and the treatment with the antiserum against the nerv growth factor (Levi-Montalcini & Angeletti, 1966), presumably because of their alread advanced degree of differentiation of birth (Angeletti & Levi-Montalcini, 1970*a*).

At the time of birth, when the drug administration was initiated, the pineal body has ver

few catecholamine-containing sympathetic fibres (Machado, 1971; unpublished observations by L. Eränkö), and the number of such fibres is small in the iris of the newborn rat (de Champlain *et al.*, 1970). Therefore, most sympathetic axons are on the way to their destination, and the still small sympathetic cells in the ganglia are intensely occupied with protein synthesis. This may partly explain the vulnerability of the sympathetic neurons of the newborn rats, although other factors, such as better penetration of the drugs to the cells and axons, may also contribute to the vulnerability of young animals.

It has been proposed that the deleterious effect of 6-hydroxydopamine on sympathetic neurons depends on the selective uptake through the axon membrane, which leads to a permanent depolarization and membrane damage (Furness *et al.*, 1970). Direct cytolytic effect of 6-hydroxydopamine was demonstrated by Angeletti & Levi-Montalcini (1970*b*) in cultures of sympathetic neuroblastoma cells. Saner & Thoenen (1971) have shown that oxidation products of 6-hydroxydopamine undergo covalent binding with biological macromolecules which leads to protein denaturation. Similar mechanisms may also explain the destructive effect of guanethidine on sympathetic neurons.

Chemical sympathectomy can thus be caused not only by treatment with 6-hydroxydopamine but also by administration of guanethidine to newborn rats, although the latter drug has much milder effects on the adult sympathetic system. This may apply to a number of other drugs whose effects on the developing nervous system are yet unknown.

References

ANGELETTI, P. U. (1971). Chemical sympathectomy in newborn animals. *Neuropharmacol.* **10,** 55–9.

ANGELETTI, P. U. & LEVI-MONTALCINI, R. (1970*a*). Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. Nat. Acad. Sci.* **65,** 114–21.

ANGELETTI, P. U. & LEVI-MONTALCINI, R. (1970*b*). Cytolytic effect of 6-hydroxydopamine on neuroblastoma cells. *Cancer Res.* **30,** 2863–9.

de CHAMPLAIN, J., MALMFORS, T., OLSON, L. & SACHS, Ch. (1970). Ontogenesis of peripheral adrenergic neurons in the rat: pre- and postnatal observations. *Acta physiol. scand.* **80,** 276–88.

COHEN, S. (1960). Purification of a nerve growth promoting protein from mouse salivary gland and its cytotoxic antiserum. *Proc. Nat. Acad. Sci., Wash.* **46,** 302–11.

ERÄNKÖ, O. (1967). The practical histochemical demonstration of catecholamines by formaldehyde induced fluorescence. *J. R. microsc. Soc.* **87,** 259–76.

ERÄNKÖ, O. & ERÄNKÖ, L. (1971*a*). Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of rat pineal after chemical sympathectomy with 6-hydroxydopamine. *Histochem. J.* **3,** 357–63.

ERÄNKÖ, L. & ERÄNKÖ, O. (1971*b*). Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat. *Acta physiol. scand.* In press.

FURNESS, J. B., CAMPBELL, G. R., GILLARD, S. M., MALMFORS, T., COBB, I. L. S. & BURNSTOCK, G. (1970). Cellular studies of sympathetic denervation produced by 6-hydroxydopamine in the vas deferens. *J. Pharmacol. exp. Ther.* **174,** 111–22.

JENSEN-HOLM, J. & JUUL, P. (1970). The effect of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: Cholinesterases and catecholamines (histochemistry), and histology. *Acta pharmacol. et toxicol.* **28,** 283–98.

LEVI-MONTALCINI, R. & ANGELETTI, P. U. (1966). Immunosympathectomy. *Pharmacol. Rev.* **18,** 619–28.

MACHADO, A. B. M. (1971). Electron microscopy of developing sympathetic fibres in the rat pineal body. *Progr. Brain Res.* **34,** 171–86.

MALMFORS, T. & SACHS, Ch. (1968). Histochemical studies on the accumulation of noradrenaline after depletion with guanethidine, metaraminol or an inhibitor of noradrenaline synthesis, H 44/68. *Europ. J. Pharmacol.* **2,** 265–75.

MITCHELL, J. R. & OATES, I. A. (1970). Guanethidine and related agents. I. Mechanism of the selective blockade of adrenergic neurons and its antagonism by drugs. *J. Pharmacol. exp. Ther.* **172,** 100–7.

PORTER, C. C., TOTARO, J. A. & STONE, C. A. (1963). Effect of 6-hydroxydopamine and some other compounds on the concentration of norepinephrine in the hearts of mice. *J. Pharmacol. exp. Ther.* **140,** 308–16.

SANAN, S. & VOGT, M. (1962). Effect of drugs on the noradrenaline content of brain and peripheral tissues and its significance. *Brit. J. Pharmacol.* **18,** 109–27.

SANER, A. & THOENEN, H. (1971). Model experiments on the molecular mechanism of action of 6-hydroxydopamine. *Molec. Pharmacol.* **7,** 147–54.

STONE, C. A., PORTER, C. C., STAVROSKI, J. M., LUDDEN, C. T. & TOTARO, J. A. (1964). Antagonisms of certain effects of catecholamine-depleting agents by antidepressants and related drugs. *J. Pharmacol. exp. Ther.* **144,** 196–204.

THOENEN, H. (1969). *Bildung und funktionelle Bedeutung adrenerger Ersatztransmitter.* Berlin, Heidelberg & New York: Springer-Verlag.

THOENEN, H. & TRANZER, J. P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **261,** 271–88.

PRINTED IN GREAT BRITAIN BY THE BROADWATER PRESS LTD, WELWYN GARDEN CITY, HERTFORDSHIRE

Acta pharmacol. et toxicol. 1971, **30**, 403–416.

From the Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki 00170, Finland

Effect of Guanethidine on Nerve Cells and Small Intensely Fluorescent Cells in Sympathetic Ganglia of Newborn and Adult Rats

By

Liisa Eränkö and Olavi Eränkö

(Received July 22, 1971; Accepted August 17, 1971)

Abstract: Newborn rats were given intraperitoneal injections of 20 mg/kg body weight of guanethidine daily for 8 days. About 3 weeks later, they were killed together with their untreated litter mate controls at the age of one month. Guanethidine caused destruction of the superior cervical and coeliac ganglia, the size of which was about 10 % of that of the control ganglia. The number of nerve cells in the ganglion remnants was also reduced. The mean number of the small intensely fluorescent (SIF) cells of the superior cervical ganglion increased from 479 cells/ganglion to 1427, and those of the coeliac ganglion from 198 to 1071 cells/ganglion, after guanethidine administration. The number of SIF cells increased in normally existing clusters from which grew out finger-like cell protrusions. Adult animals injected with 20 mg/kg body weight of guanethidine daily for 14 days and killed on the 15th day had superior cervical ganglia of slightly increased size. The space between the ganglion cell bodies was much increased and was infiltrated by numerous round-nucleated cells, the cytoplasm of which was non-fluorescent. The ganglion cells showed degenerative signs of varying degree, such as loss of basophilia and amine fluorescence. No increase in the number of SIF cells was observed. It is concluded that guanethidine causes a chemical sympathectomy accompanied by hyperplasia of the SIF cells in newborn rats, and severe degenerative changes with round cell infiltration in the sympathetic ganglia of adult rats.

Key-words: Guanethidine – sympathetic ganglia – catecholamines – chemical sympathectomy.

There are two main types of cells in the sympathetic ganglia: ordinary anglion cells, which exhibit a catecholamine fluoresecence of variable ıtensity in the cytoplasm, and small cells the fluorescence of which is very ıuch more intense than that of the most intensely fluorescent ganglion cells ERÄNKÖ & HÄRKÖNEN 1963, 1965; NORBERG & HAMBERGER 1964; VAN ORDEN *et al.* 1970; ERÄNKÖ & ERÄNKÖ 1971c). Substances causing a deple-

tion of catecholamines from the sympathetic ganglion cells, such as re serpine, α-methyl-*m*-tyrosine or α-methyl-*p*-tyrosine, have much less effect c the catecholamine content of the small intensely fluorescent (SIF) cells (V ORDEN *et al.* 1970). Moreover, prolonged administration of 6-hydroxydo amine, which in newborn mice and rats causes a complete and permane destruction of most sympathetic neurons (ANGELETTI & LEVI-MONTALCI 1970), has no effect on the appearance or number of the SIF cells (ERÄNK & ERÄNKÖ 1971a),

Guanethidine is a drug known to cause a loss of catecholamines fro sympathetically innervated tissues and sympathetic ganglia, such as t superior cervical ganglion (SANAN & VOGT 1962). Prolonged administratic of this drug has been reported to cause depletion of noradrenaline, loss cholinesterase activity, chromatolytic changes in the ganglion cells and i filtration of the superior cervical ganglion by small lymphocyte-like ce (JENSEN-HOLM & JUUL 1970a & b). These cells were reported to be no fluorescent and thus not SIF cells.

The present study was undertaken to study the effect of prolonged a ministration of guanethidine on the sympathetic ganglia of both adult a newborn rats, of which the latter are known to be more sensitive than adu rats to drugs such as 6-hydroxydopamine and guanethidine, both of whic cause destruction of sympathetic nerve fibres in the iris and the pineal bo of new born rats (ERÄNKÖ & ERÄNKÖ 1971d & e).

Material and Methods

Experimental.

The dose schedule employed for the newborn rats was similar to that used wi 6-hydroxydopamine by ANGELETTI & LEVI-MONTALCINI (1970) and ERÄNKÖ & ERÄN (1971a). Three litters of newborn rats, descendants of the Sprague-Dawley strai were divided into two equal groups, of which one was given intraperitoneal inje tions of 20 mg of guanethidine sulphate (ismelin®, kindly supplied by Ciba-Geig Basle) per kg body weight daily for eight days, the other serving as contro The first injection was given within 12 hours of birth. Guanethidine was dissolv in 0.9 % sodium chloride solution containing 2 mg of the drug per ml of solve The animals were killed by cutting the vertebral column and the dorsal aorta the age of 1 month, about 3 weeks after the last guanethidine injection. In t results these animals are referred to as "young rats".

Ten adult male rats of the Sprague-Dawley strain were injected intraperitonea with guanethidine 20 mg/kg body weight daily for 14 days. They were killed the 15th day together with 10 untreated male rats. In the study by JENSEN-HOLM JUUL (1970b) this dose caused marked ganglion cell changes.

Demonstration of catecholamines.

For the study of the distribution of histochemically demonstrable catecholamine the formaldehyde induced fluorescence method was used essentially as describ

by ERÄNKÖ (1967). Immediately after killing, the superior cervical ganglia and the coeliac ganglia were spred on an aluminium foil, frozen by immersion in propane cooled to −190° with liquid nitrogen, dried in vacuum with a phosphorus pentoxide trap for 1 week, exposed to formaldehyde gas for 30 min. at 50° and 1 hr at 80°, generated from paraformaldehyde equilibrated with 60 % atmospheric humidity, and embedded in a mixture of Epon and Araldite (ERÄNKÖ & ERÄNKÖ 1971c). The ganglia of the young rats were cut at 5 μm into complete series of sections with a glass knife using the LKB Pyramitome. The sections were transferred on slides dry with a brush. For fluorescence microscopy, a Leitz Ortholux microscope was used with a HBO 200 mercury lamp (Osram), a Ploem (1971) epiilluminator and the following filters (Schott & Gen.): 3 mm BG 38, 3 mm BG 3, 3 mm BG 12 and TAL 405 for excitation, the dichroic mirror 2 and K 470 (Leitz) filter above the objective.

The number of the small intensely fluorescent cells was counted from complete series of sections of the superior cervical ganglion and coeliac ganglion of the young rats, using the Floderus formula (ERÄNKÖ 1955) for correction of the error due to section thickness. In adult rats several mid-sections through the ganglia were examined but no counts were made.

Results

General observations.

The newborn rats injected with guanethidine showed retardation of growth, and weighed about 40 g at the time of killing, while the untreated controls weighed about 60 g. The injected rats were apparently in a poor condition, had a shaggy coat of hair, and sat in the corner of the cage. However, none of the animals died until 2 weeks after cessation of the injections, though 2 rats died during the last week of the first month. The ganglia of the 16 injected young rats and 15 controls were examined.

The adult rats injected with guanethidine showed normal growth and their general condition and behaviour did not differ from that of the untreated controls.

The sympathetic ganglia.

The size of superior cervical ganglion and the coeliac ganglion was distinctly reduced in the young rats injected with guanethidine daily for 3 days after birth and then allowed to recover for 3 weeks. The length and the diameter of the former were, however, both reduced to less than one half of the same measurements in the control ganglia, which means a volume reduction to about 10 %, or less of the original. This volume loss was due to irreversible destruction of sympathetic ganglion cells.

Fig. 1 shows a typical fluorescence photomicrograph of the superior cervical ganglion of an untreated young control rat. The ganglion cells exhibit a fluorescence the intensity of which varies from one individual

cell to another. A small cluster of SIF cells is visible in the upper righ corner; because of the intense fluorescence of these cells they have becom overexposed and therefore look less sharp.

Views through the residual nodules of the superior cervical gangli of the young rats which had been injected with guanethidine were ver variable from one visual field to another (figs. 2–4). In some regions th number of ganglion cells per unit area of section had decreased only littl (fig. 2), while in others only few nerve cell bodies were seen, the mai part of the tissue being composed of fluorescent and non-fluorescent pro cesses of SIF cells and ganglion cells, as well as of non-fluorescent glia cells (figs. 3 and 4). Many of the surviving ganglion cell bodies were ab normally large (fig. 2), and their shape and fluorescence intensity wa very variable.

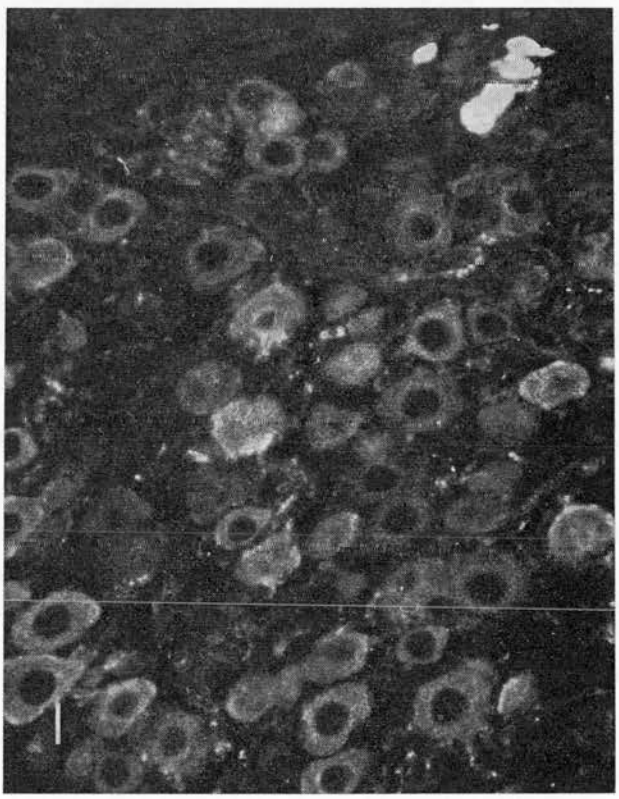


Fig. 1. Formaldehyde induced fluorescence in the superior cervical ganglion of a one month-old control rat. A small cluster of SIF cells is visible in the upper righ corner. Magnification × 350.

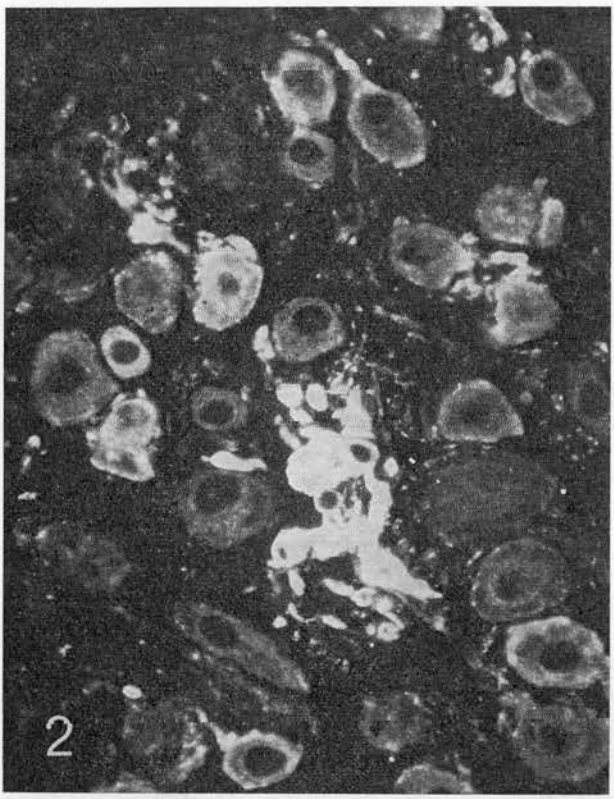


Fig. 2. Photomicrograph obtained under identical conditions of the surviving nodule in the superior cervical ganglion of a one-month-old rat given 8 daily injections of guanethidine after birth. Note the cluster of SIF cells in the centre and the large ganglion cell bodies with variable fluorescence. Magnification × 350.

The most striking feature in the residual ganglia of the guanethidine-injected young rats was the large irregular clusters of SIF cells, from which finger-like cell protrusions often grew out in a manner suggesting intense proliferative growth of the SIF cells (figs. 3 and 4). Thick intensely fluorescent processes apparently originating from the SIF cells were seen everywhere in the ganglion remnant.

Changes perhaps even more marked than those in the superior cervical ganglion were seen in the coeliac ganglion of young rats injected with guanethidine. Fig. 5 shows the coeliac ganglion of a control. Fig. 6 is a view of the coeliac ganglion of an injected rat; it is composed almost exclusively of intercellular tissue with numerous nerve fibres and a typical cluster of SIF cells.

In the superior cervical ganglia of the adult rats a distinct increase i the distance between individual ganglion cells was observed by fluorescenc microscopy, which also revealed variable loss of fluorescence from thei cytoplasm.

After fluorescence microscopy the Epon-Araldite sections were staine with toluidine blue and examined in transmitted light. Severe loss c basophilic material was seen in many of the ganglion cells. There was pronounced infiltration by numerous small round-nucleated cells in th enlarged space between the ganglion cell bodies.

Number and distribution of SIF cells.

Loss of the sympathetic ganglion cells and their processes observed i the young rats after treatment with guanethidine would in itself cause a

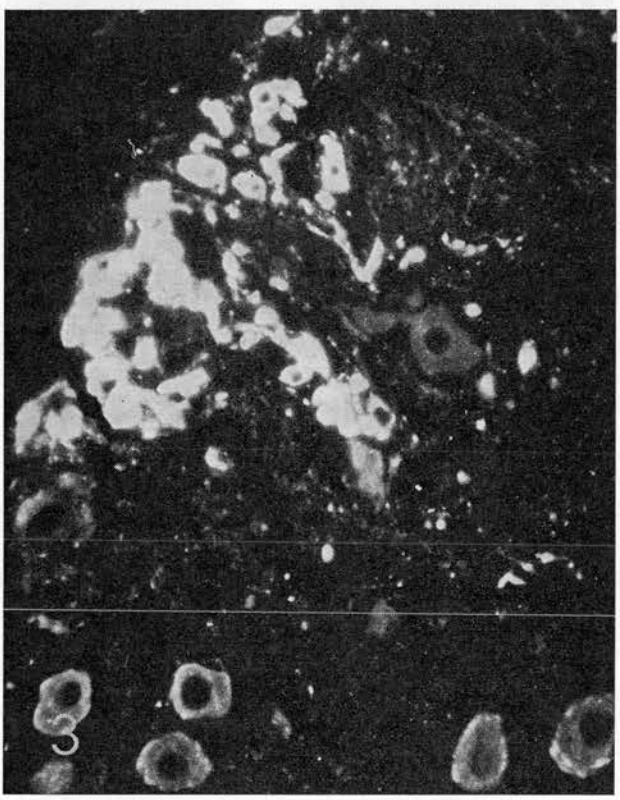


Fig. 3. Another field from the remnants of the superior cervical ganglion of a youn guanethidine-injected rat. A large cluster of SIF cells is seen in the upper left corne Note the scarcity of ganglion cell bodies. Magnification × 350.

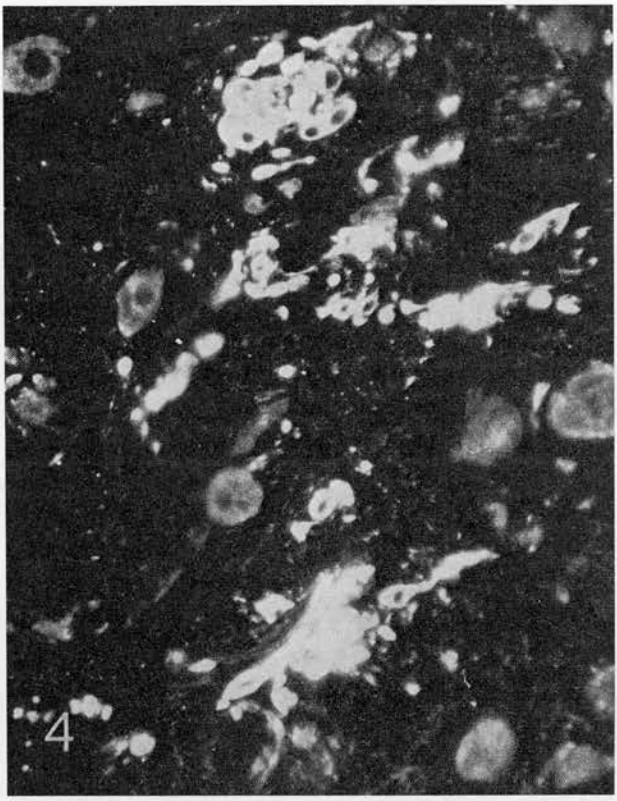


Fig. 4. Several SIF cell clusters in the same ganglion as that shown in fig. 3. Magnification × 350.

ıpparent increase in the number of SIF cells since these come closer ogether, replacing the destroyed sympathetic neurons, as has been shown o be the case after treatment with 6-hydroxydopamine (ERÄNKÖ & ERÄNKÖ 971a). The actual counting of these cells was therefore necessary, even hough visually as seen in fig. 4 they presented a picture of true pro- iferation. Table 1 shows that indeed there was a highly significant in- rease in the number of the SIF cells, which was 3 times as large in the uperior cervical ganglion and 5 times as large in the coeliac ganglion of he young rats injected with guanethidine, as the number of these cells in the ame ganglia of the untreated young rats, respectively.

Fig. 7 shows the distribution of the small intensely fluorescent cells in ıe superior cervical ganglia of a young control rat and a rat treated with uanethidine daily for 8 days after birth. The drawings were made by oing through the complete series of sections of each ganglion and by

marking on the drawing each SIF cell as a black dot. Comparison of thes two drawings suggests that the increase in the total number of SIF cel caused by guanethidine was due to an increase in the number of cel within each previously existing cluster of SIF cells. Moreover, the numbe of cell clusters may have decreased through fusion of neighbouring cluster

Few SIF cells were seen in the ganglia of adult rats injected wit guanethidine. Although no counts were made it was clear that the numbe of cells decreased rather than increased. The small round cells did nc show any amine fluorescence, as was seen by simultaneous fluorescenc microscopy by epiillumination and phase microscopy in transmitted visibl light.

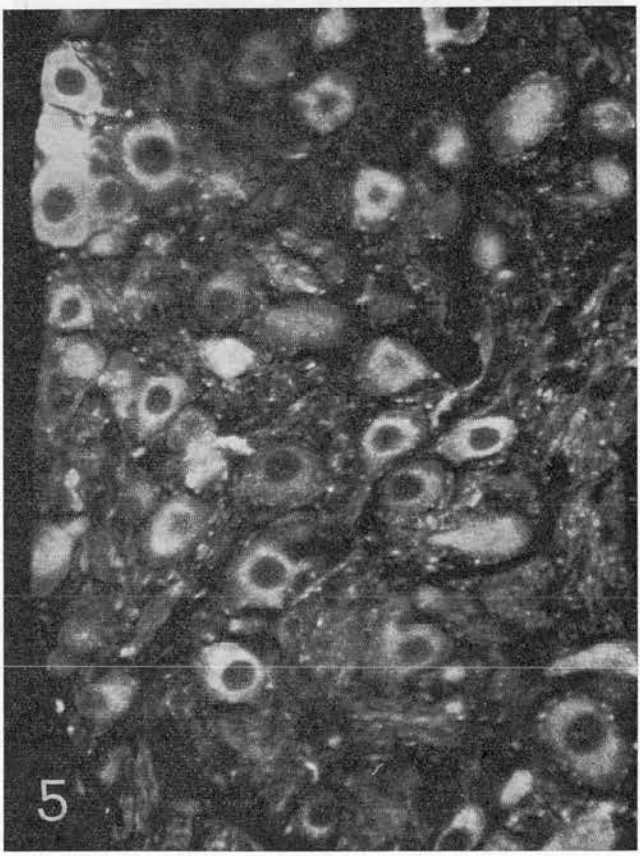
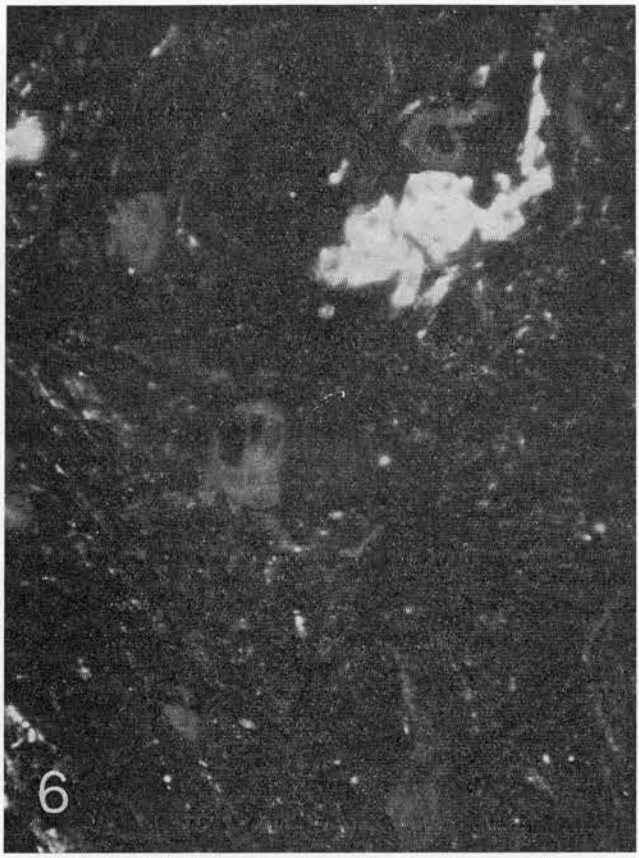


Fig. 5. Formaldehyde induced fluorescence in the coeliac ganglion of a young contr rat. Note the perinuclear cytoplasmic fluorescence typical of this ganglion and th numerous intercellular fibres with fluorescent beadings. Magnification × 350.



ig. 6. Typical field of the remnants of the coeliac ganglion in a young rat injected ith guanethidine. Only a few ganglion cell bodies are left but there are numerous uorescent nerve fibres. A SIF cell cluster is visible in the upper right corner. Magnification × 350.

Discussion

In our study, guanethidine had essentially similar effects on the superior ervical ganglion of adult rats as those previously reported by JENSEN-IOLM & JUUL (1970a & b). After the same dosage of guanethidine as that sed by us, i.e. 20 mg/kg daily for 14 days, JENSEN-HOLM & JUUL (1970b) escribed an achromatosis of the nerve cells and loss of cholinesterases. ı our study, a marked loss of cytoplasmic basophilia from the ganglion ells was observed. We also confirmed their observations on the appearance f large amounts of small lymphocyte-like cells and the somewhat larger ound cells in the superior cervical ganglion of adult rats 14 days after

guanethidine administration. JENSEN-HOLM & JUUL (1970b) pointed ou that these cells resemble the cells described by LAMPERT (1965) in ex perimental allergic encephalomyelitis, and they suggested some kind of im munological reaction as a possible reason for the round cell infiltratio In our opinion the possibility might be considered that destruction of th ganglion cells is the primary effect of guanethidine and the round ce infiltration the secondary reaction caused by dead ganglion cells.

Both in the study of JENSEN-HOLM & JUUL (1970b) and in our materia the administration of guanethidine did not cause any increase in the num ber of SIF cells of the adult rats. However, it is not quite clear whethe the small infiltrating cells, though non-fluorescent, are related to the SI cells, the number of which was greatly increased in young rats after guar ethidine administration. However, this seems unlikely as judged from th morphological properties of the infiltrating small cells and those of th SIF cells.

Table 1.

Effect of guanethidine on the number of small intensely fluorescent (SIF) cells i sympathetic ganglia of the rat. Newborn rats were injected with 20 mg/kg bod weight of guanethidine sulphate daily for 8 days. They were killed after 22 days a the age of 1 month.

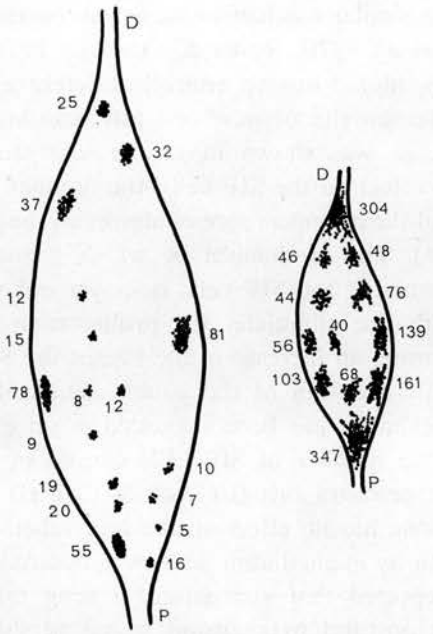
Superior cervical ganglion

Group	Number of rats	Number of SIF cells per ganglion		P*
		Mean	S.D.	
Control	7	479	70	< 0.0001
Guanethidine	5	1427	392	

Coeliac ganglion

Group	Number of rats	Number of SIF cells per ganglion		P*
		Mean	S.D.	
Control	4	198	10	~ 0.002
Guanethidine	4	1071	333	

* The value of P, indicating the probability that the difference of the means is du to random variation, has been calculated using Student's t-test.



ig. 7. Distribution and number of SIF cells in the superior cervical ganglion of wo one-month-old rats. On the left, the ganglion of an untreated control; on the ght, the ganglion of a rat injected daily for 8 days with 20 mg/kg of guanethidine. , distal; P, proximal end of the ganglion. The total number of cells in the control anglion was 441, divided into 17 clusters, that in the injected rat 1432 in 12 clusters. he drawings were made by examining complete series of sections through the ganglion.

In the present study, guanethidine caused a dramatic irreversible de-truction of the sympathetic ganglion cells in the pre- and paravertebral ympathetic ganglia when administered to newborn rats. These changes ere similar to those obtained after "chemical sympathectomy" with 6-ydroxydopamine (ANGELETTI & LEVI-MONTALCINI 1970; ANGELETTI 1971; ERÄNKÖ & ERÄNKÖ 1971a). Since guanethidine treatment also leads to estruction of the peripheral sympathetic fibres (ERÄNKÖ & ERÄNKÖ 1971), n the same way as does 6-hydroxydopamine (THOENEN & TRANZER 1968; ANGELETTI & LEVI-MONTALCINI 1970; ERÄNKÖ & ERÄNKÖ 1971d), it can e said that guanethidine causes chemical sympathectomy in newborn ats (ERÄNKÖ & ERÄNKÖ 1971e). Since it is known that guanethidine is aken up by the sympathetic nerve fibres and that it is capable of acting s a false transmitter substance, like 6-hydroxydopamine (PORTER *et al.* 963; BOULLIN 1966; THOENEN 1969), it seems reasonable to assume that

both drugs have a similar mechanism of action on the sympathetic neuron (see VAN ORDEN *et al.* 1970; SANER & THOENEN 1971).

However, guanethidine has an entirely different effect on the SIF cel in the sympathetic ganglia of newborn rats, causing a 3–5 fold increas in their number, as was shown in the present study, while 6-hydroxy dopamine has no effect on the SIF cells, the number of cells remaining un changed in spite of the disappearance of almost all the ganglion cells (ERÄNK & ERÄNKÖ 1971a). The mechanism by which guanethidine causes an in crease in the number of the SIF cells is as yet unknown. However, it ap pears that guanethidine stimulates the proliferation of previously existin SIF cells, thus causing an increase in the size of the SIF cell clusters, rathe than inducing differentiation of the young stem cells into SIF cells. Th latter type of mechanism has been suggested as an explanation for the 10 fold increase in the number of SIF cells caused by the administration o hydrocortisone in newborn rats (ERÄNKÖ & ERÄNKÖ 1971b). On the othe hand, hydrocortisone has no effect on the sympathetic ganglion cells, whic are destroyed both by guanethidine and by 6-hydroxydopamine.

It has been reported that guanacline, a drug related to guanethidine causes in man a postural hypotension which persists for several month after withdrawal of the drug (BOCK & HEIMSOTH 1969; DAWBORN *et a* 1971). Furthermore, BURNSTOCK *et al.* (1971) observed that the administra tion of guanacline sulphate (5 mg/kg/day for 9–14 weeks) in rats causes massive deposition of lipoprotein granules into the sympathetic ganglio cells, which therefore become intensely autofluorescent; no such change were observed in rats injected with the same dose of guanethidine sulphate the ganglia of which appeared essentially normal. BURNSTOCK *et al.* (1971 suggested that the postural hypotension observed in man long after cessa tion of guanacline treatment might be related to the cellular effects o guanacline. While our study shows that guanethidine sulphate (20 mg kg/day) causes serious degenerative changes in sympathetic ganglion cells no increase in autofluorescence was observed. Because of the high dose, n conclusions can be drawn from the present study concerning the toxicit of therapeutic doses of guanethidine in man.

The effects of 6-hydroxydopamine, guanethidine and hydrocortisone de pend on the administration of these substances into newborn animals an have milder effects on the sympathetic nervous system of adult animal In this respect they resemble the effects of the nerve growth factor an its antiserum (COHEN 1960; LEVI-MONTALCINI 1964; LEVI-MONTALCINI & ANGELETTI 1966). It appears, indeed, likely that many other drugs whic are known to influence the adult sympathetic nervous system might exe more profound and permanent effects when administered into newbor animals. Thus, new important information may be obtained on the forma

on, differentiation and growth of the sympathetic nervous system and ervous system as a whole.

Acknowledgements

Our best thanks for skilled technical assistance are due to Mrs. Paula Iasenson and Mrs. Tuula Stjernvall.

The present study has been supported by a grant from the Sigrid usélius Foundation.

REFERENCES

ngeletti, P. U.: Chemical sympathectomy in newborn animals. *Neuropharmacol.* 1971, **10**, 55–59.

ngeletti, P. U. & R. Levi-Montalcini: Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. Nat. Acad. Sci.* 1970, **65**, 114–121.

ock, K. D. & V. Heimsoth: Persistierende orthostatische Hypotonie nach Behandlung mit Guanacline. *Deutsch. med. Wschr.* 1969, **94**, 265–270.

oullin, D. J.: A calcium requirement for release of ^{3}H-guanethidine by sympathetic nerve stimulation. *J. Pharm. Pharmacol.* 1966, **18**, 709–712.

urnstock, G., A. E. Doyle, B. J. Cannon, J. F. Gerkens, T. Iwayama & M .L. Mashford: Prolonged hypotension and ultrastructural changes in sympathetic neurones following guanacline treatment. *Eur. J. Pharmacol.* 1971, **13**, 175–187.

ohen, S.: Purification of a nerve growth promoting protein from the mouse salivary gland and its neurocytotoxic antiserum. *Proc. nat. Acad. Sci. U.S.A.* 1960, **46**, 302–311.

awborn, J. K., A. E. Doyle, A. Ebringer, J. Howqua, G. Jerums, C. I. Johnston, M. L. Mashford & J. D. Parkin: Persistent postural hypotension due to Guanacline. *Pharmacol. Clin.* 1971, **2**, 1–5.

ränkö, L. & O. Eränkö: Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat. *Acta physiol. scand.* 1971a, in press.

ränkö, L. & O. Eränkö: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extraadrenal chromaffin tissue of the rat. *Acta physiol. scand.* 1971b, in press.

ränkö, O.: *Quantitative methods in histology and microscopic histochemistry*. Little, Brown and Comp., Boston, Toronto 1955, pp. 160.

ränkö, O.: The practical histochemical demonstration of catecholamines by formaldehyde induced fluorescence. *J. microsc. Soc.* 1967, **87**, 259–276.

ränkö, O. & L. Eränkö: Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* 1971c, **34**, 39–52.

ränkö, O. & L. Eränkö: Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of rat pineal after chemical sympathectomy with 6-hydroxydopamine. *Histochem. J.* 1971d, in press.

ränkö, O. & L. Eränkö: Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats. *Histochem. J.* 1971e, in press.

ränkö, O. & M. Härkönen: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963, **58**, 285–286.

Eränkö, O. & M. Härkönen: Monoamine-containing small cells in the superior cervic ganglion of the rat and an organ composed of them. *Acta physiol. scand.* 196 **63**, 511–512.

Jensen-Holm, J. & P. Juul: The effects of guanethidine, pre- and postganglion nerve division on the rat superior cervical ganglion: cholinesterases and protei *Acta pharmacol. et toxicol.* 1970a, **28**, 270–282.

Jensen-Holm, J. & P. Juul: The effects of guanethidine, pre- and postganglionic ner division on the rat superior cervical ganglion: cholinesterases and catecholamin (histochemistry) and histology. *Acta pharmacol. et toxicol.* 1970b, **28**, 283–29

Lampert, P. W.: Demyelination and remyelination in experimental allergic encephal myelits. Further electron microscopic observations. *J. Neuropath & Exp. Neur* 1965, **24**, 371–385.

Levi-Montalcini, R.: The nerve growth factor. *Ann. N.Y. Acad. Sci.* 1964, **11** 149–168.

Levi-Montalcini, R. & P. U. Angeletti: Immunosympathectomy. *Pharmacol. Re* 1966, **18**, 619–628.

Nordberg, K.-A. & B. Hamberger: The sympathetic adrenergic neuron. *Acta physic scand.* 1964, **63**, Suppl. 238.

Ploem, J. S.: The microscopic differentiation of the colour of formaldehyde-induce fluorescence. *Progr. Brain Res.* 1971, **34**, 27–38.

Porter, C. C., J. A. Totaro & C. A. Stone: Effect of 6-hydroxydopamine and son other compounds on the concentration of norepinephrine in the hearts of mic *J. Pharmacol. exp. Therap.* 1963, **140**, 308–316.

Sanan, S. & M. Vogt: Effect of drugs on the noradrenaline content of brain ar peripheral tissues and its significance. *Brit. J. Pharmacol.* 1962, **18**, 109–127.

Saner, A. & H. Thoenen: Model experiments on the molecular mechanism of actic of 6-hydroxydopamine. *Mol. Pharmacol.* 1971, **7**, 147–154.

Thoenen, H.: *Bildung und funktionelle Bedeutung adrenerger Ersatztransmitter.* Spri ger-Verlag, Berlin, Heidelberg, New York 1969, pp. 85.

Thoenen, H. & J. P. Tranzer: Chemical sympathectomy by selective destruction adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedebergs Arc exp. Path. Pharmak.* 1968, **261**, 271–288.

Van Orden, L. S., J. P. Burke, M. Geyer & F. V. Lodoen: Localization of depletio sensitive and depletion-resistant norepinephrine storage sites in autonomic gangli *J. Pharmacol. exp. Ther.* 1970, **174**, 56–71.

Acta physiol. scand. 1972. 84. 115—124

From the Department of Anatomy, University of Helsinki, Finland

Effect of 6-Hydroxydopamine on the Ganglion Cells and the Small Intensely Fluorescent Cells in the Superior Cervical Ganglion of the Rat

By

Liisa Eränkö and Olavi Eränkö

Received 8 July 1971

Abstract

Eränkö, L. and O. Eränkö. *Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat.* Acta physiol. scand. 1972. *84*. 115—124.

Newborn rats were injected daily for 8 days with 6-hydroxydopamine, (6-OH-DA) 50 mg/kg b. w. Disappearance of over 90 % of the ganglion cells from the superior cervical ganglion and the coeliac ganglion was observed 3 weeks after discontinuation of the injections. The surviving ganglion cells were larger than normal and exhibited less intense than normal catecholamine fluorescence but showed normal or slightly decreased activity of acetylcholinesterase and non-specific cholinesterase. Electron microscopically, hypertrophy of the endoplasmic reticulum and the Golgi apparatus were observed. 6-OH-DA caused, besides destruction of ganglion cells, an increase in the relative number of the small intensely fluorescent cells. However, this increase was due to loss of ganglion cells from between the small cells, and the total number of small intensely fluorescent cells counted from complete series of sections through the ganglia was the same in controls as that in animals injected with 6-OH-DA. It is concluded that the small intensely fluorescent cells are resistant to chemical sympathectomy, and differ in this respect from ordinary ganglion cells.

Administration of 6-hydroxydopamine (6-OH-DA) causes, in an adult animal, a reversible destruction of peripheral adrenergic nerve terminals without affecting the nerve cell body (Tranzer and Thoenen 1967, 1968). In newborn animals 6-OH-DA has been reported to cause a life-long chemical sympathectomy, *i.e.*, irreversible degeneration of the peripheral adrenergic nerve terminals and permanent destruction of sympathetic ganglia (Angeletti and Levi-Montalcini 1970).

Different types of sympathetic ganglion cells have been described on the basis of their cholinesterase activity and catecholamine fluorescence (see Härkönen 1964, Eränkö 1967 a); moreover, small intensely fluorescent (SIF) cells are present in the sympathetic ganglia whose properties differ from those of ordinary ganglion cells (Eränkö and Härkönen 1963, 1965; see also reviews by Jacobowitz 1970 and Eränkö and Eränkö 1971 a). To our knowledge, neither cholinesterases and catecholamines

nor SIF cells have been examined in sympathetic ganglia of animals treated with 6-OH-DA. Such a study is reported in this paper, together with some observations on the changes in the fine structure of the ganglion cells.

Material and Methods

Experimental

Newborn rats were injected within 12 h after birth intraperitoneally with 6-OH-DA a gift kindly supplied by Messrs. F. Hoffmann-La Roche, Basel) 50 mg/kg b.w.; a similar injection was thereafter given daily in the following 7 days, a dosage schedule proposed by Angeletti and Levi-Montalcini (1970) for complete destruction of nerve cells from all sympathetic ganglia of the rat. A fresh solution was prepared immediately before each injection by dissolving 5 mg of 6-OH-DA in 1 ml of 0.9 % sodium chloride solution containing 0.2 mg of ascorbic acid in each ml.

Of each litter, one half of the rats were injected, while the other half served as untreated controls. The injected rats, altogether 16 animals, were killed together with the same number of untreated litter mate controls at the age of about 1 month, when 3 weeks had elapsed after the last injection of 6-OH-DA. The superior cervical and coeliac ganglia were removed immediately after killing.

Demonstration of catecholamines

The fresh ganglia were placed on an aluminium foil and frozen on it by immersion in propane cooled to −190° C with liquid nitrogen. Freeze-drying was carried out for a week at −45° C in vacuum, using a phosphorus pentoxide trap close by the tissue holder. After warming of the tissues to 40° C under vacuum, air was let into the apparatus. The dry ganglia were then transferred into a petri dish containing paraformaldehyde powder which had been equilibriated with 60 % relative air humidity. The petri dish was then placed in an incubating owen kept at 50° C for 30 min and warmed then to 80° C, at which temperature the exposure was further continued for 1 h. For details of the technique the reader is referred to Eränkö (1967 b).

The formaldehyde-treated ganglia were embedded in a mixture of Epon and Araldite (Eränkö and Eränkö 1971 a) and cut into complete series of 5 μm thick sections with a glass knife, using the LKB Pyramitome. The sections were transferred dry on a slide and mounted in Entellan (E. Merck, Darmstadt). For fluorescence microscopy of catecholamines, an Ortholux microscope (E. Leitz, Wetzlar) was with an Osram HBO 200 high-pressure mercury lamp, two 3 mm thick BG 38 filters, one 3 mm thick BG 3 and one TAL 405 interference filter (Schott et Gen., Mainz), and with the Ploem (1971) epiilluminator with dichroic mirrors. Above the objective, the ultraviolet absorbing filter K 470 (Leitz) was used.

For counting of the SIF cells, all sections cut from the superior cervical ganglia were examined. The section thickness error was corrected using the Floderus formula (see Eränkö 1955).

Demonstration of cholinesterases

The ganglia were fixed by immersion as whole in an ice-cold 3.5 % solution of formaldehyde in 0.1 M phosphate buffer, pH 7.4. The formaldehyde was freshly made by dissolving paraformaldehyde with sufficient dropwise addition of sodium hydroxide solution to bring the paraformaldehyde into solution. After fixation overnight, the ganglia were rinsed, again overnight, in 0.1 M phosphate buffer, pH 7.4, with 0.3 M of sucrose. Thereafter, frozen sections were cut at 10 μm. The sections were incubated immersed in the substrate mixture containing acetylthiocholine and made according to Gomori's (1952) transcription of Koelle's (1951) original recipe.

Before incubation, the sections were pre-incubated for 30 min in 10^{-5} M solution of tetraisopropylpyrophosphoramide (iso-OMPA, L. Light, Colnbrook), a specific inhibitor of non-specific cholinesterase (butyrylcholinesterase), when acetylcholinesterase was demonstrated. The same inhibitor was included in the same concentration in the incubation solution containing the substrate.

For the demonstration of non-specific cholinesterase, the pre-incubation and incubation were carried out in the same way but using, instead of iso-OMPA, 1 : 5-bis-(4-allyl dimethylammoniumphenyl) pentan-3-one diiodide (284C51, Burroughs and Wellcome, London), a specific inhibitor of acetylcholinesterase, in a concentration of 10^{-5} M, and buturylthiocholine as a substrate. The efficiency of the inhibitors towards the cholinesterase activities in the sympathetic ganglia was tested and the above concentrations proved suitable (see also Eränkö *et al.* 1964).

Electron microscopy

Small pieces of the ganglia were cut with a razor blade before fixation for electron microscopy. The pieces were then immersed in the fixatives which were kept at 0° C in tubes immersed in granular ice. The fixatives used were (1) 3.5 % potassium permanganate dissolved in a phosphate-buffered Krebs-Ringer solution, pH 7.0, which is a modification of the permanganate fixative first proposed by Richardson (1964); (2) 2.5 % glutaraldehyde and 1 % formaldehyde in 0.1 M phosphate buffer, pH 7.4, according to Karnovsky (1965).

Fixation was carried out for 1 h in permanganate and the tissues were then rinsed overnight in the Krebs-Ringer solution at 0° C, dehydrated and embedded in a mixture of Epon and Araldite (Eränkö and Eränkö 1971 a). After fixation in the glutaraldehyde-formaldehyde mixture, the tissues were rinsed overnight in the Krebs-Ringer solution at 0° C, re-fixed in an 1 % solution of osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 0° C for 1 hour, rinsed in 0.1 M phosphate buffer for 30 min, as well as dehydrated and mounted in the Epon-Araldite mixture as described above. Thin sections cut with the LKB Ultrotome were examined with the Philips EM 300 electron microscope at 40 or 60 kV.

Results

General observations

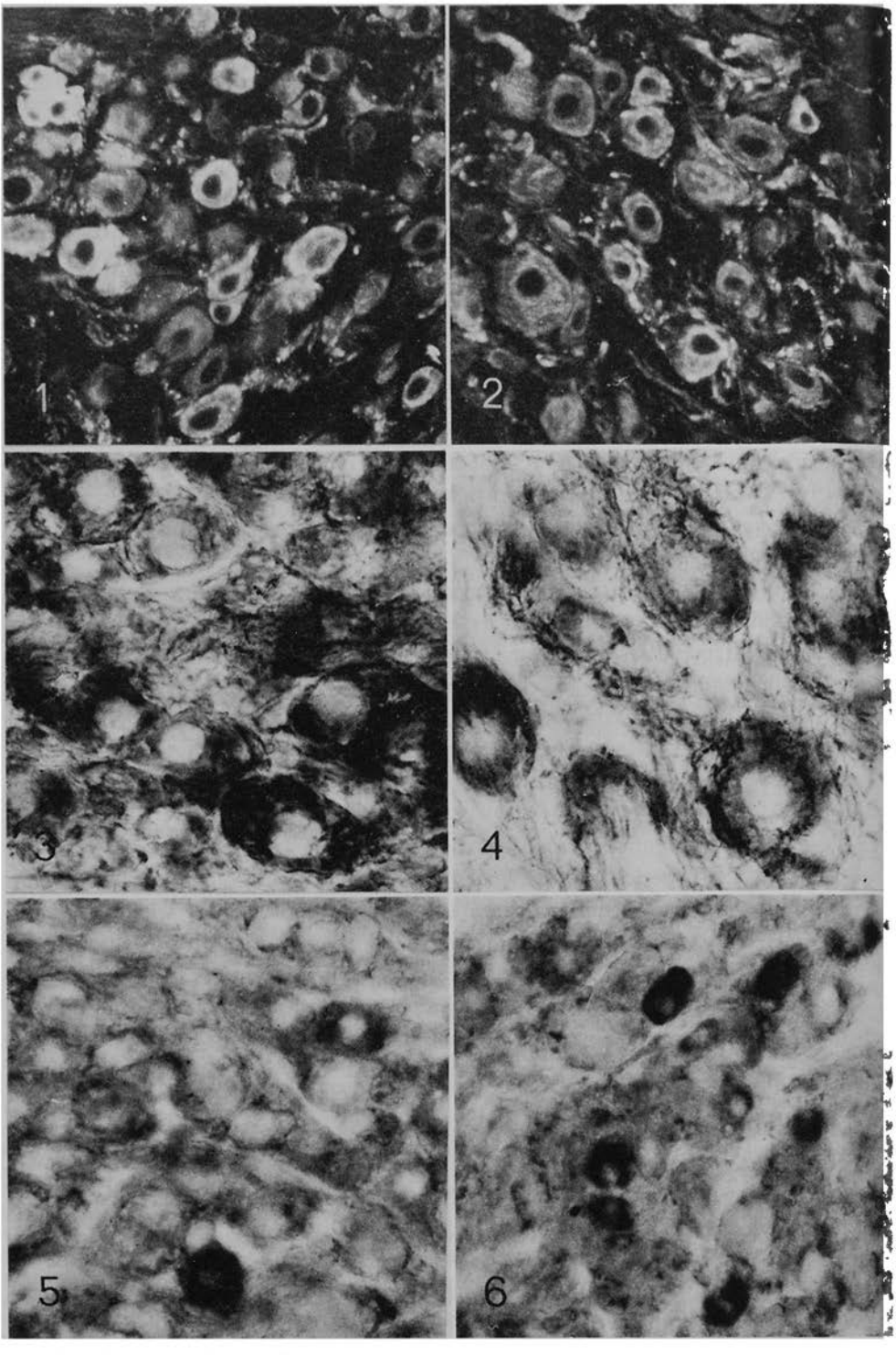
The general appearance of the rats which had been injected with 6-OH-DA was essentially similar as that of their untreated litter mate controls. However, they weighed about 20 % less than the controls. No differences in the behaviour or spontaneous activity were observed.

The size of the superior cervical ganglion and that of the coeliac ganglion were drastically reduced in the animals injected with 6-OH-DA. The diameter of the superior cervical ganglion of injected rats was reduced to less than one third of original, which indicates a volume diminution to less than about one tenth of the control value. In some of the 6-OH-DA injected animals, small rounded nodules were observed in the slender ganglia of the injected rats.

Catecholamine fluorescence

Fig. 1 shows the distribution of the formaldehyde induced fluorescence, due to noradrenaline, in the superior cervical ganglion of an 1-month-old control rat. The ganglion cell bodies exhibited a diffuse cytoplasmic fluorescence, whose intensity varied from one ganglion cell to another. In addition to the diffuse cytoplasmic fluorescence, fluorescent granules were seen in the peripheral parts of the perikaryon. In the normal ganglia, the cells were fairly close to each others, although there was a distinct intercellular space between the cell bodies, filled with both non-fluorescent and fluorescent cell processes. A small group of SIF cells is also visible in the left upper corner of Fig. 1.

Fig. 2 is of an identically made preparation from the nodular part of the superior cervical ganglion of an 1-month-old rat injected daily for 8 days with 50 mg of hydroxydopamine per kg b.w. The general view is not much different from that of the normal ganglion illustrated in Fig. 1, although most of the cells exhibit only a relatively weak fluorescence, the variation between the individual cell bodies being thus smaller than in normal controls. The relative amount of intercellular tissue was distinctly increased, and several abnormally large cells were seen. One such cell is above the number 2 in Fig. 2. Since over 90 % of the original ganglion had com-



(For legends see opposite page.)

TABLE I. Effect of 6-hydroxydopamine on the number of small intensely fluorescent (SIF) cells in the superior cervical ganglion of the rat.

Group	Number of rats	SIF cells per ganglion	
		Mean	S.D.
Control	5	466	76
6—OH—DA	5	472	68

pletely disappeared after treatment of 6-OH-DA, it is understandable that large areas of the sympathetic trunk were also seen in the injected animals in which no ganglion cells were seen at all.

The relative number of the SIF cells in the ganglion appeared greatly increased after treatment with 6-OH-DA. However, the increase was found to be due to concentration of the SIF cells into a smaller volume, when most ordinary ganglion cells, which normally separate the clusters of small cells, had become destroyed by the 6-OH-DA treatment. When the total number of small intensely fluorescent cells was calculated from a complete series of sections, about 470 cells were found both in the controls and in the injected animals (Table I).

Cholinesterases

Acetylcholinesterase (AChE). The distribution of AChE activity in a normal ganglion is illustrated in Fig. 3. The variation in the AChE activity between individual cell bodies is evident, and the fine cholinesterase positive nerve fibres are clearly visible between the cell bodies.

A ganglion from a rat injected with 6-OH-DA is shown in Fig. 4. Except for a slight increase in the relative amount of intercellular tissue, comparison with Fig. 3 also suggests an increase in the average size of the nerve cell bodies and some loss of AChE activity of the cell bodies. The rich AChE positive nerve net between the cell bodies is clearly visible.

Fig. 1. Formaldehyde-induced fluorescence in the superior cervical ganglion of a control rat. Individual ganglion cell bodies are close to each others and show a fluorescence of variable intensity. 3 SIF cells are visible in the left upper corner. × 250.

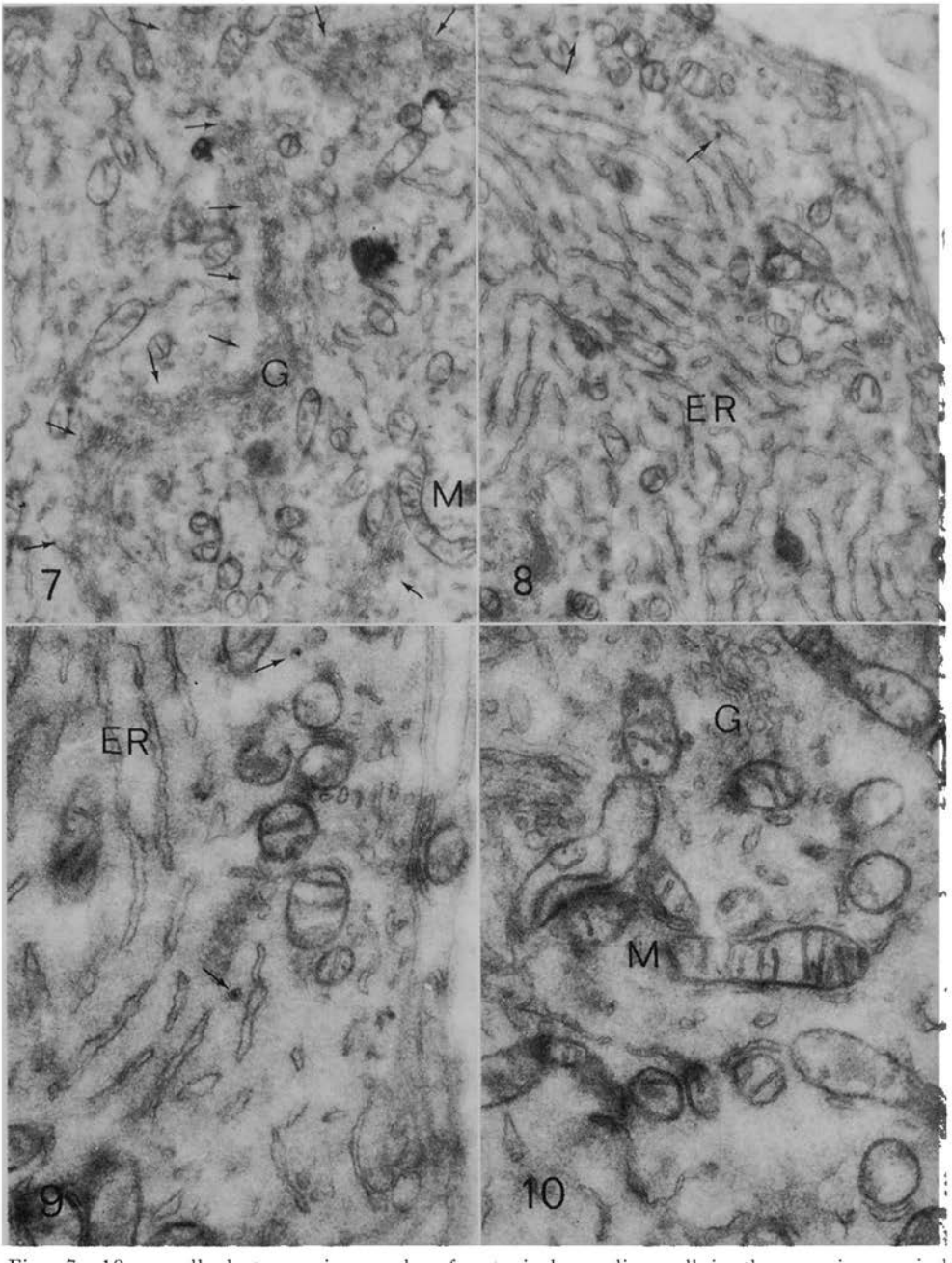
Fig. 2. Similar photograph of a rat injected after birth with 6-OH-DA. Observe large distances between the ganglion cell bodies. A hypertrophic cell visible on the lower left part of the figure. × 250.

Fig. 3. AChE in the superior cervical ganglion of a control rat. Note the variation in the activity between individual ganglion cells. × 400.

Fig. 4. Similar field as that in Fig. 3 of a rat treated with 6-OH-DA. The cells appear larger and they show a weakened acetylcholinesterase activity. Beaded nerve fibres are especially clearly visible in the wide intercellular spaces and on the nerve cell bodies. × 400.

Fig. 5. Non-specific cholinesterase activity in the superior cervical ganglion of a control rat. Most cell bodies show a weak reaction but there is one intensely reactive cell in the lower centre. × 250.

Fig. 6. Non-specific cholinesterase in the same ganglion of a rat treated with 6-OH-DA. The view is essentially similar as that in Fig. 5, save for the coincidental presence of 5 intensely reactive cells. × 250.



Figs. 7—10 are all electron micrographs of a typical ganglion cell in the superior cervical ganglion of a rat treated with 6-OH-DA. Permanganate fixation, no counterstain, voltage 40 kV.

Fig. 7. Part of perinuclear cytoplasm with a well-developed Golgi apparatus (G) indicated by arrows. M, mitochondrion. × 20,000.

Fig. 8. Another field of the periphery of the same cell, showing the extensive endoplasmic reticulum (ER) whose numerous ribosomes are not demonstrable with this fixation. Arrows indicate two granular vesicles. × 20,000.

Non-specific cholinesterase. The distribution of non-specific cholinesterase was essentially similar in the ganglia of 6-OH-DA-treated rats (Fig. 6) as that in the ganglia of untreated control rats (Fig. 5), although the increase in the size of the cell bodies and in the relative amount of intercellular tissue was again apparent. Since it has been shown that cells exhibiting an intense activity of non-specific cholinesterase correspond to cells exhibiting a weak catecholamine fluorescence (Härkönen 1964), special attention was paid to the number of such cells, possibly cholinergic ones, in the ganglia of 6-OH-DA-treated rats. However, no significant differences could be observed, as compared with normal controls.

Electron microscopy

In the normal control rats aged one month, the nerve cells of the superior cervical ganglion showed all signs of intense synthetic activity. The nuclear membrane was often folded and fitted with several nuclear pores. The Golgi apparatus and the granular endoplasmic reticulum were well developed. In the peripheral areas of the cytoplasm there were clusters of small granular vesicles. Between the cell bodies, axons were visible embedded in the Schwann cell cytoplasm and containing alternatively empty vesicles or small granular vesicles.

Because of the great variation in the size and the fine structural appearance between individual ganglion cells of normal rats, it was difficult to find features in the ganglia of the rats treated with 6-OH-DA which had been specifically due to drug administration. It was quite clear that whatever cells had survived, they also showed well-developed and perfectly normal cell organelles. Moreover, the impression was obtained that in the ganglia of the 6-OH-DA-treated animals there were signs of an increased cellular activity. The endoplasmic reticulum seemed to be more extensive in many cells (Fig. 8) and the Golgi apparatus appeared more prominent (Fig. 7). However, as compared with the control ganglion, less small granular vesicles were found in the periphery of the cytoplasm (Fig. 8 and 9). All mitochondria (Fig. 10) showed normal cristae but they were perhaps longer than those in the control cells. Mitochondrial swelling was never encountered in the ganglia of the injected animals, and the cristae mitochondriales were well developed.

Discussion

The results of the present study show that administration of 6-OH-DA dopamine in newborn rats daily for 8 days causes a disappearance of almost all sympathetic ganglion cells from the superior cervical ganglion and the coeliac ganglion. This is in agreement with the observation by Angeletti and Levi-Montalcini (1970), whose study was carried out mainly with mice, although it was briefly mentioned that similar results were obtained with newborn rats.

Fig. 9. A detail of the same cell shown in Fig. 8. The peripheral rim of the cell contains mitochondria and two granular vesicles (arrows). × 40,000.

Fig. 10. A view from the central part of the same cell showing typical large mitochondria (M) with intact cristae. Part of a Golgi apparatus (G) is also visible. × 40,000.

However, our results differ from those obtained by Angeletti and Levi-Montalcini (1970) in so far that not all cells had become destroyed, nodules of obviously living ganglion cells being observed in all rats 3 weeks after discontinuation of the 6-OH-DA-treatment. In these animals no such degenerative lesions were seen as were described by Angeletti and Levi-Montalcini in the neurons of the injected mice, such as disappearance of cell membranes, alteration in the mitochondria, and lacunar spaces scattered throughout the cytoplasmic area, changes which they observed soon after treatment with 6-OH-DA. Since our material dealt with rats 3 weeks after cessation of the treatment, it is indeed likely that changes which had eventually occurred during the treatment of 6-OH-DA even in the surviving nerve cells had already disappeared during the recovery period.

The ganglion cells which survived the treatment with 6-OH-DA differed somewhat from those of untreated controls. As judged from the formaldehyde induced fluorescence, the catecholamine content of the perikaryon was smaller than that in the controls. The size of the cells was increased, and there was a tendency towards hypertrophy of the endoplasmic reticulum and the Golgi apparatus as well as an increase in the number of mitochondria. These are signs which can also be observed after division of the axon. It is therefore of special interest that the changes described by Angeletti and Levi-Montalcini (1970) at the early stages of 6-OH-DA-treatment, such as mitochondrial swelling, have been observed in the early state of axon reaction (Härkönen 1964). This is noteworthy because 6-OH-DA has a more potent action on the axon terminals than the cell body, which in adult animal remains unaffected by the treatment. The discussed changes in the cell body of animals which have been injected with 6-OH-DA since birth, suggest that the principal site of its action may be the axon, which then causes an axon reaction in the cell body, which in some cases is permanent and leads to cell destruction but which is in the minority of cells reversible and compatible with survival and re-innervation of peripheral organs such as the iris (Eränkö and Eränkö 1971 b). However, it seems likely that 6-OH-DA also directly affects the ganglion cell body, especially in the newborn rats, whose ganglion cells are yet at a poorly differentiated stage. The mechanism proposed by Furness *et al.* (1970) for the peripheral axon membrane, *i.e.* uptake of 6-OH-DA until a critical concentration is reached and the axon membrane is damaged, can be expected to apply also to the nerve cell membrane, which is in newborn animals not only less differentiated but also less protected by Schwann cells than in adult ganglia.

It is of interest to note that a small nerve cell population ranging in size from 2 to 10 % of the controls persists in sympathetic ganglia of newborn animals "immunosympathectomized" by injections of antiserum against the nerve growth factor (Levi-Montalcini and Angeletti 1966), and these authors propose "that these para- and prevertebral sympathetic neurons owe their resistance to the antiserum to the fact that they have already attained a considerable degree of differentiation when the treatment was first initiated". The size of the cell population which we observed to survive the treatment with 6-OH-DA was essentially similar, and the same ex-

planation can be given for its persistence, knowing that permanent effects of 6-OH-DA on sympathetic ganglion cells are also dependent on sufficiently early administration (see the introduction).

It is of special interest that the number of SIF cells in the sympathetic ganglia remains completely unaffected, showing neither increase nor decrease after treatment with 6-OH-DA. This shows, firstly, that 6-OH-DA does not affect the SIF cells in the same way as it does most of the sympathetic ganglion cells, may be because the SIF cells are more differentiated. Secondly, it indicates that the destruction of the sympathetic nervous system to the degree of over 90 % does not lead to compensatory hyperplasia of these cells, whose number dramatically increases after similar treatment with guanethidine, which also causes a chemical sympathectomy closely resembling that obtained with 6-OH-DA (Eränkö and Eränkö 1971 c). Angeletti and Levi-Montalcini (1970) reported hypertrophy of the adrenal medulla two weeks after discontinuation of treatment with 6-OH-DA, whose chromaffin cells closely resemble the SIF cells of the sympathetic ganglia (Eränkö and Eränkö 1971 a).

The present study was undertaken after a stimulating discussion with Dr. Marthe Vogt, for which the authors are very grateful.

Skilful technical assistance by Mrs. Paula Hasenson and Mrs. Tuula Stjernvall is gratefully acknowledged.

This work was supported by a grant from the Sigrid Jusélius Foundation (to O.E.).

Addendum

After the present manuscript was completed the report by Angeletti (1971) was brought to our attention. In agreement with our results, he observed 2 weeks and 3, 6 or 10 months after 6-OH-DA administration small nodules with some nerve cells in the pre- and paravertebral sympathetic ganglia of mice and rats, instead of the complete destruction reported in the earlier paper by Angeletti and Levi-Montalcini (1970).

References

Angeletti, P. U., Chemical sympathectomy in newborn animals. *Neuropharmacol.* 1971. *10.* 55—59.

Angeletti, P. U. and R. Levi-Montalcini, Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. nat. Acad. Sci.* (Wash.) 1970. *65.* 114—121.

Eränkö, O., *Quantitative Methods in Histology and Microsopic Histochemistry.* Little, Brown and Comp., Boston, Toronto 1955.

Eränkö, O., Histochemistry of nervous tissues: catecholamines and cholinesterases. *A. Rev. Pharmacol.* 1967 a. *7.* 203—222.

Eränkö, O., The practical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. Roy. micr. Soc.* 1967 b. *259—276.*

Eränkö, O. and L. Eränkö, Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* 1971 a. *34.* 39—52.

Eränkö, O. and L. Eränkö, Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of rat pineal after chemical sympathectomy with 6-hydroxydopamine. *Histochem. J.* 1971 b. In press.

Eränkö, L. and O. Eränkö, Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats. *Acta pharmacol.* (Kbh.) 1971 c. In press.

ERÄNKÖ, O. and M. HÄRKÖNEN, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58*. 285—286.

ERÄNKÖ, O. and M. HÄRKÖNEN, Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* 1965. *63*. 511—512.

ERÄNKÖ, O., M. HÄRKÖNEN, A. KOKKO and L. RÄISÄNEN, Histochemical and starch gel electrophoretic characterization of desmo- and lyoesterases in the sympathetic and spinal ganglia of the rat. *J. Histochem. Cytochem.* 1964. *12*. 570—581.

FURNESS, J. B., G. R. CAMPBELL, S. M. GILLARD, T. MALMFORS, J. L. S. COBB and G. BURNSTOCK, Cellular studies of sympathetic denervation produced by 6-hydroxydopamine in the vas deferens. *J. Pharmacol. exp. Ther.* 1970. *174*. 111—122.

GOMORI, G., *Microscopic Histochemistry*. Chicago University Press, Chicago 1952.

HÄRKÖNEN, M., Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. *Acta physiol. scand.* 1964. *63*. Suppl. 237.

JACOBOWITZ, D., Catecholamine fluorescence studies of adrenergic neurons and chromaffin cells in sympathetic ganglia. *Fed. Proc.* 1970. *29*. 1929—1944.

KARNOVSKY, M. J., A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. cell. Biol.* 1965. *27*. 137—138.

KOELLE, G. B., The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol. exp. Ther.* 1951. *103*. 153—171.

PLOEM, J. S., The microscopic differentiation of the colour of formaldehyde-induced fluorescence. *Progr. Brain. Res.* 1971. *34*. 27—38.

RICHARDSON, K. C., The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles. *Amer. J. Anat.* 1964. *114*. 173—206.

TRANZER, J. P. and H. THOENEN, Ultramorphologische Veränderungen der sympathischen Nervenendigungen der Katze nach Vorbehandlung mit 5- und 6-Hydroxy-Dopamin. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1967. *257*. 343—344.

TRANZER, J. P. and H. THOENEN, An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia* (Basel) 1968. *24*. 155—156.

KUNGL. BOKTR. STHLM 1972

Acta physiol. scand. 1972. 84. 125—133

From the Department of Anatomy, University of Helsinki, Finland

Effect of Hydrocortisone on Histochemically Demonstrable Catecholamines in the Sympathetic Ganglia and Extra-adrenal Chromaffin Tissue of the Rat

By

Liisa Eränkö and Olavi Eränkö

Received 8 July 1971

Abstract

Eränkö, L. and O. Eränkö. *Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat.* Acta physiol. scand. 1972. *84.* 125—133.

Hydrocortisone acetate was administered intraperitoneally to newborn rats daily for 5 days, 40 mg/kg b.w. They were killed on the 6th day. Adult rats were given the same dose daily for 14 days and killed on the 16th day. Catecholamines were demonstrated by formaldehyde vapour induced fluorescence. In the 6-day-old rats treated with hydrocortisone there was a distinct increase in the size of the extra-adrenal chromaffin bodies, in the number of fluorescent cells in them and in the intensity of the fluorescence of these cells, as compared with the controls. Hydrocortisone treatment also caused about a tenfold increase in the number of the small intensely fluorescent (SIF) cells in the superior cervical ganglion, the coeliac ganglion and the lumbar prevertebral ganglia of the young rats. The newly formed SIF cells were scattered single or in clusters of a few cells throughout the ganglia, including sites in which no SIF cells were normally present before hydrocortisone treatment. In the adult rats, hydrocortisone did not cause any dramatic changes in the sympathetic ganglia. The SIF cells were present in clusters in the same way as in the control ganglia but there was a tendency towards an increase in the number of SIF cells in the clusters. It is concluded that hydrocortisone causes in young rats a greatly increased formation of the SIF cells from poorly differentiated, weakly fluorescent stem cells, while proliferation of already existent SIF cells is less pronounced.

Administration of cortisone or hydrocortisone to newborn rats was observed by Lempinen (1964) to cause a pronounced hyperplasia of the extra-adrenal chromaffin tissue, which normally degenerates within 2 weeks after birth. He used fixation in the mixture of potassium dichromate and formalin for the demonstration of catecholamines in the chromaffin cells. With the aid of this reaction supplemented with the Schmorl reaction, Lempinen (1964) found numerous reactive cells also in the sympathetic ganglia of the young rats treated with cortisone or hydrocortisone, although no or few chromaffin cells were observed in the sympathetic ganglia of the untreated rats.

While the effect of adrenal cortical steroids on the chromaffin tissue has been confirmed chemically by paper chromatography (Eränkö, Lempinen and Räisänen 1966)

and by quantitative catecholamine determinations (Roffi and Margolis 1966, see also the recent review by Pohorecki and Wurtman 1971), the appearance of small chromaffin cells in the sympathetic ganglia has not been investigated since. However, such a study is of obvious interest, because small cells have been observed even in normal sympathetic ganglia which exhibit an intense formaldehyde induced fluorescence due to catecholamines (Eränkö and Härkönen 1963, 1965). These small intensely fluorescent (SIF) cells, although they do not give the chromaffin reaction, have been observed to possess structural features similar to those of the truly chromaffin cells (Matthews and Raisman 1969, Eränkö and Eränkö 1971a).

The present study, in which catecholamines have been studied in the sympathetic ganglia and the extra-adrenal chromaffin tissue with the aid of formaldehyde induced fluorescence, shows that hydrocortisone has indeed dramatic effects on the SIF cells of the sympathetic ganglia.

Material and Methods

Experimental

Newborn rats were injected i.p. with hydrocortisone daily for 5 days. Hydrocortisone acetate (kindly supplied by N. V. Organon) was dissolved immediately before injection in 0.9 % sodium chloride solution to make a concentration of 4 mg/ml. The daily dose was 40 mg/kg of b.w., *i.e.* 0.4 mg to a 10 g rat. They were killed by cutting the back at the level of the heart on the 6th day after birth,together with untreated litter mate controls. While the weight of the controls increased from about 10 g at birth to about 12 g on the 16th day, the weight of the injected rats, which all had a diarrhea, decreased from about 10 to 8 g. Two litters, each divided into 6 experimental and 6 control animals were examined, or together 12 injected rats and 12 controls.

Adult male rats weighing about 200 g were injected i.p. with 40 mg per kg of b. w. of hydrocortisone acetate daily for 14 days, using the same solution as for the young rats. They were killed on the 15th day. The weight of the injected animals decreased during the treatment from 200 g to about 190 g, while that of the controls increased to about 250 g. The adrenal weight of the injected animals decreased to about one half of that of the controls. 10 injected rats and 10 controls were studied.

Demonstration of catecholamines

Immediately after killing, the superior cervical ganglia and, in the young rats, the retroperitoneal tissue block between the kidneys and the adrenals over the vertebral column were removed, placed on an aluminium foil and frozen by immersion in propane cooled to — 190° C with liquid nitrogen. Thereafter they were freeze-dried for 1 week at — 45° C in vacuum, warmed, removed from the freeze-drying apparatus and exposed to formaldehyde vapour generated from paraformaldehyde powder equilibriated with 60 % relative air humidity; for details see Eränkö (1967). After exposure for 30 min at 50° C and 1 hour at 80° C to formaldehyde, the tissues were embedded in a mixture of Epon and Araldite (Eränkö and Eränkö 1971 a), cut dry with the LKB Pyramitome at 5 μm into a complete series of sections. These were transferred dry with a brush on slides and mounted in Entellan (E. Merck, Darmstadt). Fluorescence was examined and photographed using the Ortholux microscope (E. Leitz, Wetzlar) fitted with an epiilluminator (Ploem 1971) and the following filters (Schott & Gen., Mainz) after the Osram HBO 200 lamp: 3 mm BG 38, 3 mm BG 3, TAL 405, K 470.

Fig. 1. Formaldehyde induced fluorescence in the retroperitoneal tissue block of a 6-day-old control rat. A, aorta; V, vena cava; LG, lumbar sympathetic ganglion; OZ, Organ of Zuckerkandl. × 60.

Fig. 2. Similar field as Fig. 1 of a 6-day-old rat injected daily after birth with hydrocortisone. Note the numerous fluorescent cells in the lumbar ganglion (LG) and the very intense fluorescence of the Organ of Zuckerkandl (OZ). × 60.

Fig. 3. Lumbar ganglia of a rat injected with hydrocortisone. The ganglia contain numerous fluorescent cells. × 120.

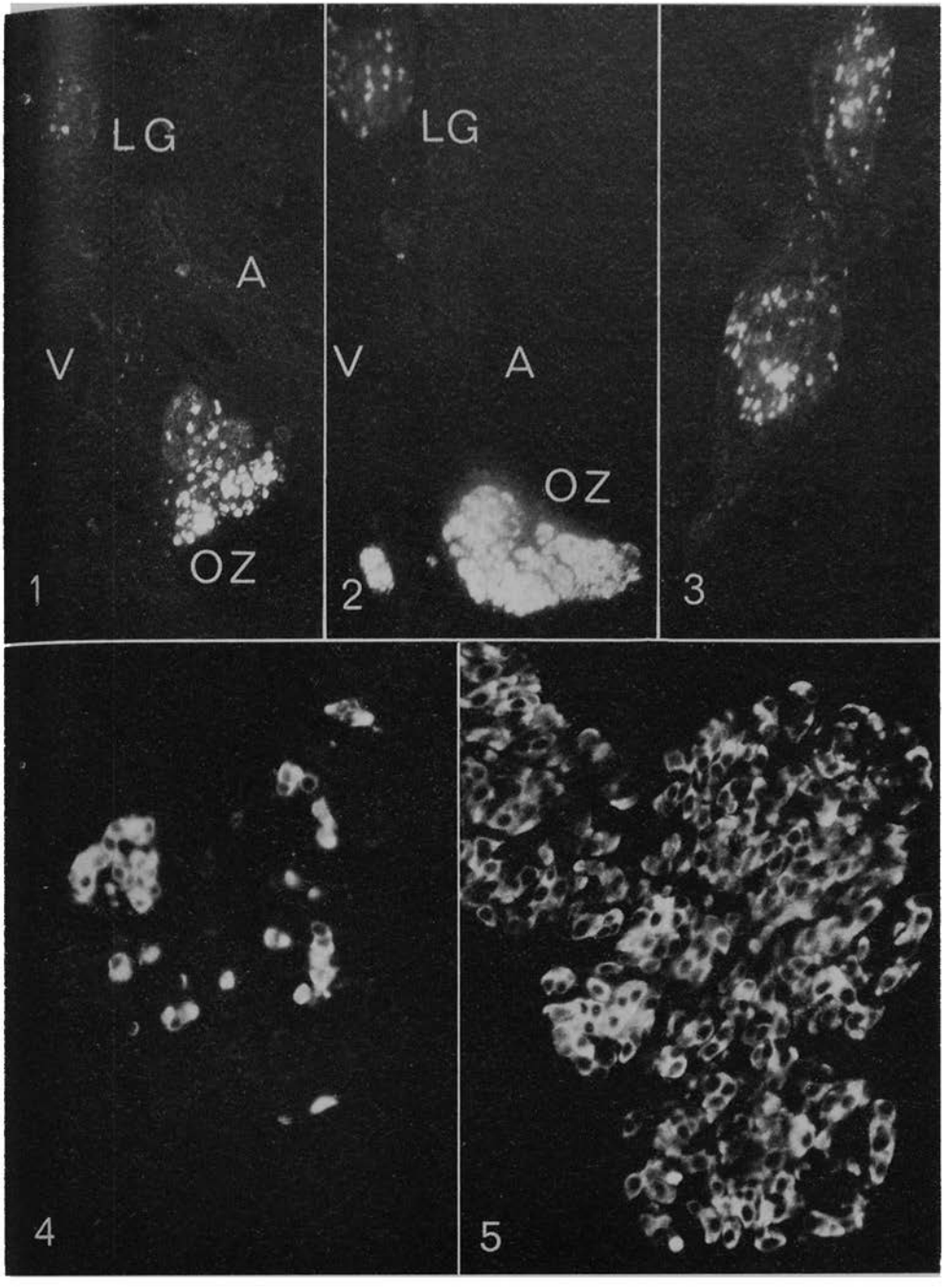


Fig. 4. The Organ of Zuckerkandl of a control rat. The photomicrograph is underexposed to comply with the very intense fluorescence of the small cells. The larger weakly fluorescent cells (see Fig. 1) are therefore hardly visible. ×250.

Fig. 5. The Organ of Zuckerkandl of a hydrocortisone injected rat. Conditions of photography as in Fig. 5. The body is mainly composed of small intensely fluorescent cells.

Results

A cross section of the retroperitoneal tissue block of a normal young (6-day-old) control rat is illustrated in Fig. 1. A weak formaldehyde induced fluorescence is visible in the lumbar ganglion (LG), in which 2 somewhat more intensely fluorescent cells are also seen. The main para-aortic body, the organ of Zuckerkandl (OZ) exhibited a more intense fluorescence than that in the ganglion, mainly because of the presence of small intensely fluorescent cells scattered amongst less intensely fluorescent cells which were more concentrated in the central end of the body. This distribution of the cells exhibiting monoamine fluorescence closely corresponded to the distribution of chromaffin cells demonstrated by fixation in dichromate-formalin mixture in the study by Lempinen (1964), as is evident by comparing his Fig. 34 with our Fig. 1. Fig. 4 is a fluorescence photomicrograph taken at a higher magnification of the OZ of another control rat. Clusters of intensely fluorescent cells are seen against the background of less fluorescent cells.

The retroperitoneal tissues of a 6-day-old rat injected daily with hydrocortisone injections since birth are illustrated in Fig. 2, made under similar conditions of preparation and photomicrography as Fig. 1. Two main differences can be seen, which were typical of all the young hydrocortisone-treated rats examined. Firstly, the num-

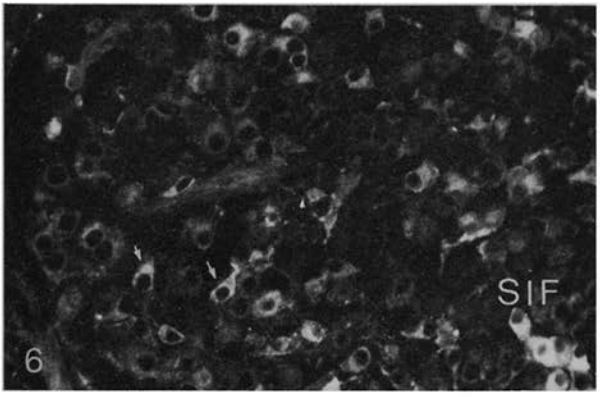


Fig. 6. Amine fluorescence in the superior cervical ganglion of a control rat. Note the 2 moderately fluorescent cells marked with arrows amongst relatively weakly fluorescent young sympathetic cells and the small intensely fluorescent cells (SIF). ×250.

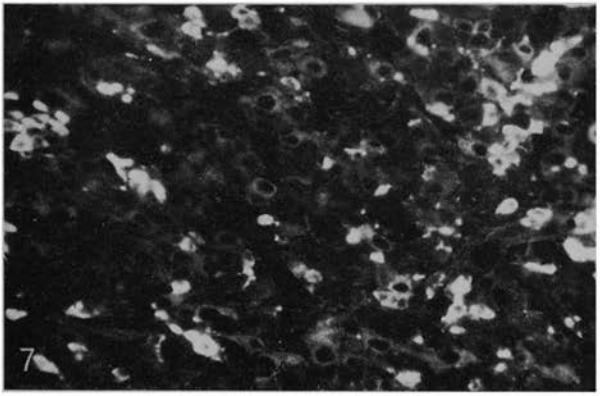


Fig. 7. Similar fluorescence photograph of the same ganglion of a rat injected with hydrocortisone. There are numerous SIF cells all over the field. ×250.

ber of SIF cells in the sympathetic ganglia, also shown in Fig. 3, was much increased as compared with the controls. Secondly, the whole OZ was filled with cells whose fluorescence intensity appeared to be even more intense than that in similar cells observed in the control rats. These cells are better shown in Fig. 4 and 5, which have been exposed less than Fig. 1—3 to avoid "washing out" of the details due to the intense fluorescence as in Fig. 2.

The monoamine fluorescence of the superior cervical ganglion of a young control rat is illustrated in Fig. 6. The ganglion cells were at the age of 6 days still small and closely packed because of the relatively small amount of structures such as dendrites, axons and Schwann cells between them. The intensity of the cytoplasmic fluorescence varied considerably between individual ganglion cells. Most cells exhibited a weak, though clearly discernible fluorescence, which was located especially around the nucleus. About one tenth of all the cells, 2 of which are indicated by arrows in Fig. 6, exhibited a moderate fluorescence. Further cells, which were rare, showed a fairly intense fluorescence. They were therefore considered SIF cells. However, it should be pointed out that the fluorescence of these cells was less intense than that in the SIF cells in the sympathetic ganglia of normal adult rats and very much less intensely fluorescent than the cells of the OZ or adrenal medulla.

After treatment for 5 days with hydrocortisone, a dramatic increase in the number of SIF cells was observed in the superior cervical ganglion of young rats, as can be seen in Fig. 7. Comparison with Fig. 6 shows that while most young ganglion cells had remained similar to those in the control ganglion, numerous clusters of SIF cells appeared everywhere in the ganglion. To obtain an idea of the order of magnitude of the increase in the number of SIF cells, these were counted from a complete series of sections from one control ganglion and one ganglion from a hydrocortisone treated rat. In the control ganglion 328 SIF cells were found, while 3110 cells were found after treatment with hydrocortisone. Although no further cell counts were made, because these were so tedious owing to the large number of evenly scattered cells, visual examination of the complete series of sections in all control and experimental animals clearly showed that the increase in the number of SIF cells was of the same order in all young injected rats.

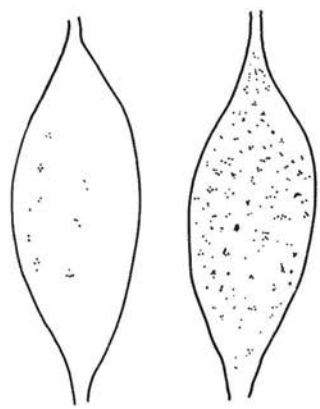


Fig. 8. Schematic drawing showing the distribution of SIF cells in typical mid-sections of the superior cervical ganglia of two 6-day-old rats. On the left, the ganglion of an untreated control rat with 25 SIF cells. On the right, the ganglion of a rat injected since birth daily with hydrocortisone; there are 259 SIF cells in the mid-section. Note the even distribution of the newly formed SIF cells after hydrocortisone treatment.

The distribution patterns of the SIF cells were also studied. It proved useless to draw a composite picture of the distribution of SIF cells in the ganglion of young hydrocortisone injected rats from a complete series of sections, the whole ganglion being covered by the SIF cells, but comparison of the midsections drawn in Fig. 8 clearly illustrate the effect of hydrocortisone. New SIF cells covered the whole ganglion in a single mid-section, while only some areas show SIF cells in the mid-section of the control ganglion. It is therefore probable that new SIF cells had been formed from less intensely fluorescent cells. Cells with a moderate cytoplasmic fluorescence such as the two cells marked with arrows in Fig. 6 were seen everywhere even in the normal sympathetic ganglion of young rats, and thus can be considered as cells potentially capable of developing into SIF cells. However, it is likely that other even less fluorescent and less differentiated cells may serve as stem cells for the SIF cells which were formed under the influence of hydrocortisone.

Preliminary electron microscopic study of the superior cervical ganglion of 6-day-old rats treated with hydrocortisone known from fluorescence microscopy to contain large numbers of SIF cells failed to demonstrate cells with numerous small granular vesicles, typical of the SIF cells of adult animals. No such cells were found after examination areas large enough to contain several SIF cells. All cells showed electron microscopic signs of intense cytoplasmic activity: the nuclear membrane was fenestrated, the endoplasmic reticulum the Golgi apparatus and the mitochondria were well developed and numerous. Although some granular vesicles of about 50 nm in diameter were seen in the axons between the cell bodies, granular vesicles were extremely rare in the cytoplasm of the young ganglion cells. Further studies are required to characterize the fine structure of the SIF cells formed after hydrocortisone treatment.

There was no difference discernible by fluorescence microscopy in the appearances of the superior cervical ganglion of the adult hydrocortisone treated rats and those of the controls. The distribution and number of the SIF cells remained essentially unaffected. In some injected animals there were clusters with increased numbers of SIF cells but the changes were not significant. Other ganglia or para-aortic tissues were not studied in the adult rat.

Discussion

The observation made in the present study that administration of hydrocortisone in newborn animals causes a hyperplasia of the extra-adrenal chromaffin tissue and the appearance of numerous SIF cells in the sympathetic ganglia confirm with fluorescence microscopical techniques the observations made by Lempinen (1964), who used the Schmorl reaction after dichromate fixation to demonstrate catecholamine-containing cells. As was also noted in his study, the effect of hydrocortisone depended on a sufficiently early beginning of the administration of cortical hormone, which had no essential effect on the adult sympathetic ganglia.

The effect of some other drugs causing alterations in the sympathetic ganglia also depend on beginning the administration before the animals are too old. Thus, in-

jections of antiserum against the nerve growth factor almost totally destroy almost all sympathetic nerve cells when given to a newborn mouse of rat but have little effect on the sympathetic ganglion of adult animals (Levi-Montalcini and Angeletti 1966). The same applies to "chemical sympathectomy" with 6-hydroxydopamine (Angeletti and Levi-Montalcini 1970) or with guanethidine (Eränkö and Eränkö 1971b, c). It appears plausible that the degree of differentiation of the ganglion cells increases resistance against the destructive mechanisms.

On the other hand, it has been observed that guanethidine, which destroys young nerve cells, causes a proliferation of the SIF cells in the sympathetic ganglia of newborn rats, as does hydrocortisone, but has no such effect in adult rats, again like hydrocortisone (Eränkö and Eränkö 1971 c). However, their mechanisms of action resulting in the SIF cell proliferation need not be the same. Indeed, hydrocortisone and guanethidine cause different types of SIF cell increase. While hydrocortisone administration results in a wide-spread new-formation of SIF cells throughout the ganglion, guanethidine causes an increase in the number of SIF cells within the clusters of SIF cells which are normally present in untreated rats of the same age (Eränkö and Eränkö 1971 c). It seems that hydrocortisone has a true inductive action leading to the formation of SIF cells from previously undifferentiated stem cells which have still a potency to become a sympathicoblast or a phaeochromoblast (or SIF cell stem cells). This view was also presented by Lempinen (1964). On the other hand, guanethidine appears to cause the proliferation of already differentiated SIF cells.

Some caution is necessary in drawing this conclusion, because the rats of the present study were killed immediately after cessation of the hydrocortisone treatment, at the age of 6 days, while the guanethidine-treated rats were permitted to recover for 3 weeks after 8 daily drug injections and killed at the age of one month (Eränkö and Eränkö 1971 c). The formation of clusters of SIF cells is more advanced in one-month-old normal rats than in 6-day-old rats. While further studies are therefore desirable, it seems from Lempinen's (1964) study that the small catecholamine-containing cells formed because of cortisone administration, remain scattered in the ganglion without formation of clusters (see his Fig. 90 of the ganglion of a two-month-old rat).

It should also be emphasized that the "SIF cell" is a descriptive term of wide content. Therefore, there may be SIF cells whose other properties are different, although they share the small size and the intense fluorescence characterizing the SIF cells. Our preliminary electron microscopic observation failed to provide evidence for the expected presence of small granular vesicles in the cytoplasm of the SIF cells newly formed after hydrocortisone treatment, while granular vesicles similar to those of the chromaffin cells are typical features of adult SIF cells (Eränkö and Härkönen 1965, Matthews and Raisman 1969). Such vesicles caused Siegrist *et al.* (1968) to call these cells chromaffin cells, although in the rat they are non-chromaffin, *i.e.* they do not become coloured after dichromate fixation alone. The presence a high concentration of catecholamine in the cytoplasm of the new SIF cells, which is beyond

reasonable doubt proved by the intense formaldehyde induced fluorescence in spite of the absence of electron microscopically demonstrable granular vesicles, suggests that high concentrations of catecholamines may be present in a cytoplasmic pool outside special storage organelles like the granular vesicles. Such a diffuse pool was proposed by Eränkö and Härkönen (1963) on the basis of fluorescence microscopic appearances of the ganglion cells of the superior cervical ganglion, the fluorescence being in part evenly distributed over the cytoplasm, in part localized in granules. Aggregates of granular vesicles were recently electron microscopically demonstrated by Van Orden *et al.* (1970) in the region of the fluorescent granules of the sympathetic ganglion cells. Our preliminary observations (unpublished) on normal adult ganglion cells suggest that the catecholamine stores exhibiting a diffuse perinuclear fluorescence correspond to the endoplasmic reticulum.

Even if further investigations are required to make clear the properties of the different types of SIF cells, their proliferation and new formation after administration of steroids such as hydrocortisone is an interesting phenomenon, which offers new possibilities for understanding the nature of the SIF cells. Moreover, it raises interesting new questions of the role of adrenocortical hormones in the normal differentiation and function of sympathetic and other nerve cells.

The authors wish to thank Mrs. Paula Hasenson and Mrs. Tuula Stjernwall for valuable technical assistance.

This work has been supported by a grant from the Sigrid Jusélius Foundation to O. E.

References

Angeletti, P. U. and R. Levi-Montalcini, Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. nat. Acad. Sci.* (Wash.) 1970. *65*. 114—121.

Eränkö, O., The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. roy. micr. Soc.* 1967. *87*. 259—276.

Eränkö, O. and L. Eränkö, Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* 1971 a. *34*. 39—52.

Eränkö, O. and L. Eränkö, Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats. *Histochem. J.* 1971 b. In press.

Eränkö, L. and O. Eränkö, Effect of guanethidine on nerve cells in sympathetic ganglia of newborn and adult rats. *Acta pharmacol.* (Kbh.) 1971 c. In press.

Eränkö, O. and M. Härkönen, Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963. *58*. 285—286.

Eränkö, O. and M. Härkönen, Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* 1965. *63*. 511—512.

Eränkö, O. M. Lempinen and L. Räisänen, Adrenaline and noradrenaline in the Organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. *Acta physiol. scand.* 1966. *66*. 253—254.

Lempinen, M., Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones in it. *Acta physiol. scand.* 1964. *62*. Suppl. 231.

Levi-Montalcini, R. and P. U. Angeletti, Immunosympathectomy. *Pharmacol. Rev.* 1966. *18*. 619—628.

Matthews, M. R. and G. Raisman, The ultrastructure and somatic efferent synapses of small granula-containing cells in the superior cervical ganglion. *J. Anat.* (Lond.) 1969. *105*. 255—282.

Ploem, J. S., The microscopic differentiation of the colour of formaldehydeinduced fluorescence. *Progr. Brain Res.* 1971. *34*. 27—38.

Pohorecky, L. A. and R. J. Wurtman, Adrenocortical control of epinephrine synthesis. *Pharmacol. Rev.* 1971. *23*. 1—35.

ROELL, J. and F. MARGOLIS, Syntèse d'adrénaline dans le tissue chromaffine extrasurrénalien, chez le Rat nouveau-né, sous l'effet de l'hydrocortisone ou de la corticostimuline. *C. R. Acad. Sci.* (Paris) 1966. *263*. 1496—1499.

SIEGRIST, G., M. DOLIVO, Y. DUNANT, C. FOROGLOU-KERAMEUS, FR. DE RIBAUPIERRE and CH. ROULLIER, Ultrastructure and function of the chromaffin cells in the superior cervical ganglion of the rat. *Ultrastruct. Res.* 1968. *25*. 381—407.

VAN ORDEN, L. S., J. P. BURKE, M. GEYER and F. V. LODOEN, Localization of depletion-sensitive and depletion-resistant norepinephrine storage sites in autonomic ganglia. *J. Pharmacol. exp. Ther.* 1970. *174*. 56—71.

KUNGL. BOKTR. STHLM 1972

Histochemical Journal, **4** (1972), 49–58

Hydrocortisone-induced increase in the number of small intensely fluorescent cells and their histochemically demonstrable catecholamine content in cultures of sympathetic ganglia of the newborn rat

O. ERÄNKÖ,* L. ERÄNKÖ,** C. E. HILL *and* G. BURNSTOCK

Department of Zoology, University of Melbourne, Parkville, Victoria, Australia

Received 15 November 1971

Synopsis. It is known that hydrocortisone causes a great increase in the number of small intensely fluorescent (SIF) cells in the sympathetic ganglia when injected into newborn rats. The effect of hydrocortisone on nervous tissue *in vitro* has not been studied previously.

Pieces of newborn rat sympathetic ganglia were cultivated in Rose chambers. Hydrocortisone was dissolved in the medium in concentrations of 1–9 mg/l. Both control and hydrocortisone-containing cultures were examined daily by phase-contrast microscopy, and the catecholamines were demonstrated histochemically by formaldehyde-induced fluorescence after 7 days in culture.

All cultures showed outgrowths of axons and supporting cells elements, although these were less extensive in the groups of cultures with hydrocortisone. After a week, SIF cells with a green fluorescence were observed in the control explants. In all cultures with hydrocortisone, a concentration-dependent increase was observed in the fluorescence intensity and the number of the SIF cells in the explant; numerous SIF cells were also seen in the outgrowth. Some SIF cells showed processes and the longest processes were seen in cultures with the highest concentration of hydrocortisone.

It is concluded that hydrocortisone causes an increased synthesis of catecholamines in the SIF cells *in vitro*, and an increase in their number by affecting either their division or their differentiation from a more immature form, or both. This effect was a direct one and not mediated by any system other than the ganglion itself. Induction of enzyme synthesis by hydrocortisone is proposed as an explanation of the increase in catecholamine concentration.

* University of Melbourne Senior Research Fellow, September 1971–August 1972; permanent address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland.
** Sunshine Foundation and Rowden White Trust Overseas Research Fellow in the University of Melbourne, September 1971–August 1972; permanent address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland.

Introduction

Sympathetic ganglia have been shown to contain, in addition to nerve cell bodies exhibiting a moderate formaldehyde-induced fluorescence due to catecholamines, some small intensely fluorescent (SIF) cells with a high concentration of monoamines (Eränkö & Härkönen, 1963, 1965*a*; Norberg & Hamberger, 1964), which microspectrofluorometric studies suggest to be dopamine (Björklund *et al.*, 1970) or noradrenaline (Eränkö & Eränkö, 1971*d*). Although the SIF cells do not usually exhibit a positive chromaffin reaction, their fine structure closely resembles that of adrenal medullary cells in that they contain intensely osmiophilic granular vesicles (Eränkö & Härkönen, 1965; Grillo, 1966; Matthews & Raisman, 1969; Williams & Palay, 1969; and others).

The responses of the SIF cells differ from those of the ganglion cells under experimental conditions. The SIF cells are not affected by pre- or postganglionic nerve division (Eränkö & Härkönen, 1965*b*) and they are less susceptible to the amine-depleting action of several drugs (Van Orden *et al.*, 1970). Chemical sympathectomy in newborn rats with 6-hydroxydopamine does not affect the number or fluorescence of the SIF cells (Eränkö & Eränkö, 1971*b*). Guanethidine, on the other hand, which causes chemical sympathectomy both in newborn and in adult rats, results in an hyperplasia of the SIF cells in the sympathetic ganglia of newborn rats (Eränkö & Eränkö, 1971*c*) but does not apparently affect the SIF cells of adult rats (Burnstock *et al.*, 1971).

The most dramatic effects on SIF cells as yet have been obtained with hydrocortisone, which caused about a tenfold increase in the number of SIF cells in sympathetic ganglia of newborn rats (Eränkö & Eränkö, 1971*a*). SIF cells have recently been demonstrated in cultures of sympathetic ganglia (Chamley *et al.*, 1972*b*).

To our knowledge, no studies have been reported on the effect of hydrocortisone on nervous tissue cultured *in vitro*. Hence, the present study was undertaken, and hydrocortisone was found to cause an even more powerful effect on the SIF cells *in vitro* than *in vivo*.

Material and methods

The tissue culture method followed that described in detail by Chamley *et al.* (1972*a*). Newborn albino rats of the Sprague-Dawley strain served as the source of sympathetic ganglia. The thoracic sympathetic chain was dissected aseptically under a binocular dissecting microscope and placed into Hanks balanced salt solution (Hanks & Wallace, 1949) with 10% (v/v) foetal calf serum, 100,000 i.u./l penicillin sodium G, 100 mg/l streptomycin sulphate and 0.2 g/l Phenol Red as a pH indicator. Adherent fat and connective tissue were carefully removed with watchmaker's forceps, and the ganglia were cut out from the interganglion connectives. The pure ganglia were cut into small pieces, placed on coverslips previously coated with collagen, covered with strips of dialysing cellophane (Visking Co., Division of Union Carbide, Chicago, U.S.A., size 27/32, average pore radius 24 Å) and cultured in modified Rose (1954) chambers.

Control chambers were supplied with Medium 199 (Salk *et al.*, 1954) supplemented with 20% (v/v) foetal calf serum, 50 i.u./l of insulin, 100,000 i.u./l penicillin sodium G and an extra 5 g/l glucose. Hydrocortisone sodium succinate was added to the culture medium to obtain final hydrocortisone concentrations of 1, 3 or 9 mg/l. The chambers were kept at 37°C in an incubator which was supplied with a constant flow of 5% carbon

dioxide in air bubbled through water. The medium was changed daily and the growth examined by phase-contrast microscopy (Zeiss Standard RA microscope).

After a week, the cultures were removed by opening the chamber and carefully peeling off the cellophane strip covering them. They were gently rinsed with a few drops of serum-free Hanks balanced salt solution and dried overnight in a vacuum desiccator over phosphorus pentoxide. The coverslips with the dry cultures on them were then transferred to another desiccator containing paraformaldehyde powder known to be in equilibrium with about 60% relative air humidity. The desiccator was closed with clamps and kept in an incubator at 80°C for 1 hr. The coverslips were immediately thereafter removed and inverted on slides on which was a drop of liquid paraffin. The fluorescence induced by formaldehyde was examined in a Leitz Ortholux microscope fitted with an HBO 200 mercury burner and a 3 mm BG 38, 3 mm BG 12 and K 530 mm filters, as well as a dark ground condenser. A Leitz Orthomat automatic microscope camera was used for taking photomicrographs, recording the length of exposure with a stop watch in order to obtain an idea of the total fluorescence output of the specimen.

Results

Appearance of cultures

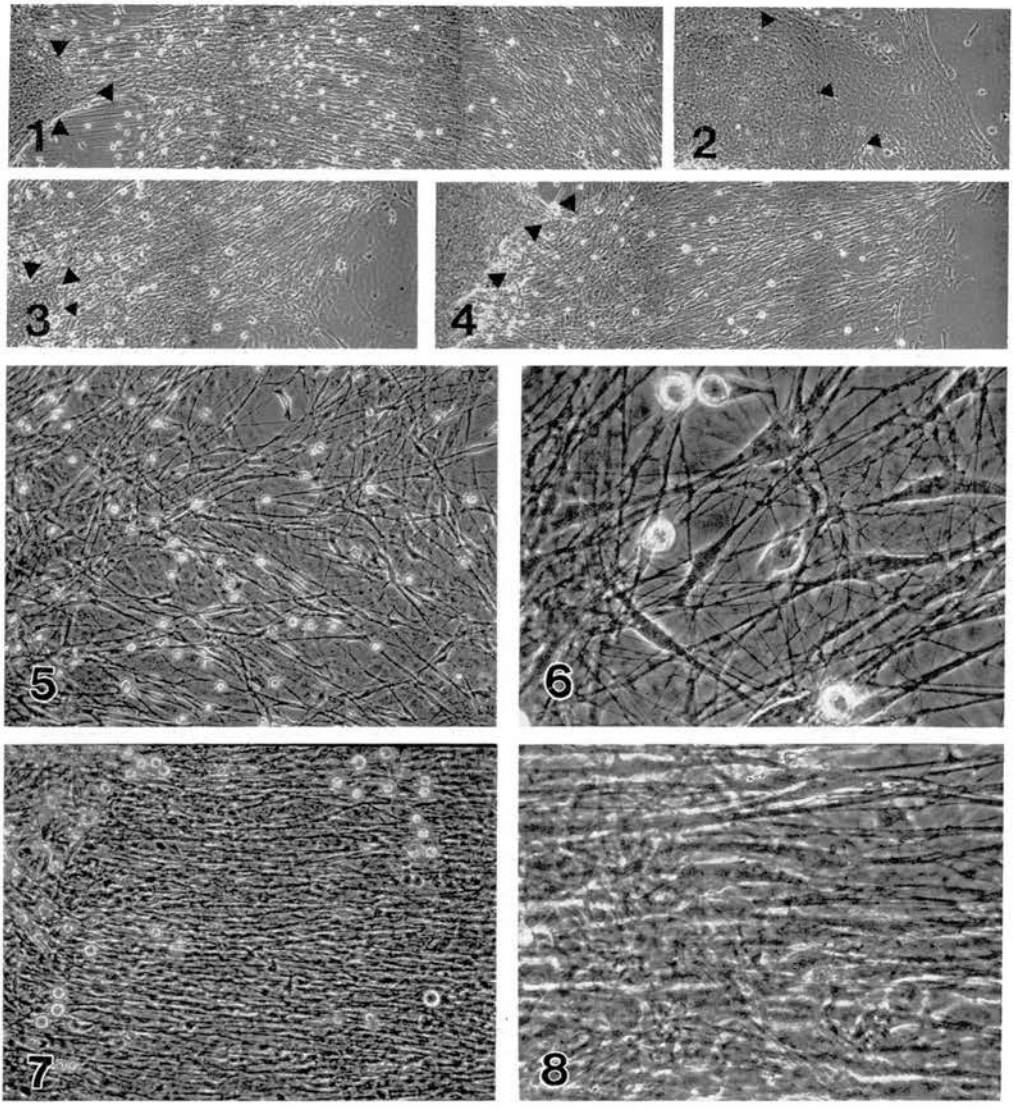
Control medium. Outgrowth of nerve fibres and supporting cells, presumably glial cells and Schwann cells, was observed after 2 days. After a week, an extensive outgrowth with crossing fibres had taken place (Figs. 1, 5 and 6). The explants were somewhat flattened, and in some cultures it was possible to recognize a few nerve cells in the outgrowth near the explant.

Hydrocortisone. While outgrowth of nerve fibres and cellular elements similar to that in the control medium was observed in all cultures with hydrocortisone, the extent of this outgrowth was more limited. The inhibition did not show any dependence on the concentration of hydrocortisone (compare Figs. 2, 3 and 4 with Fig. 1). The appearance of the outgrowth was somewhat different in the hydrocortisone-containing cultures. There were more areas with nerve fibres and Schwann cells running in a dense, parallel fashion (Figs. 7 and 8), while relatively loose outgrowth with crossing nerve fibres and flattened supporting cells was typical of the control cultures (Figs. 5 and 6). Similar dense areas were also seen in the control cultures but to a lesser extent.

Hydrocortisone had no significant effect on the size of the ganglion explants, as compared with the control cultures. The cultures with hydrocortisone showed no cellular abnormalities. The neurons, nerve fibres, Schwann cells and glial cells appeared normal, as did the number of regenerated axons.

Distribution of catecholamines

Control. In addition to autofluorescence of an orange brown colour, mainly in randomly scattered macrophages, a specific green formaldehyde-induced fluorescence was observed only in small cells considered to be SIF cells on the basis of fluorescence microscopy, although the fluorescence was not very intense (Fig. 9). The ganglion cells in the explant failed to show the typical green catecholamine fluorescence visible in normal freeze-dried ganglia. It is possible that this failure is mainly due to the simple method of



Figures 1–8 are phase-contrast photomicrographs of seven day cultures of newborn rat sympathetic ganglia. Original explants on the left are marked with triangles.

Figure 1. Control culture. × 40 *Figure* 2. Culture with 1 mg/l hydrocortisone. × 40 *Figure* 3. Culture with 3 mg/l hydrocortisone. × 40 *Figure* 4. Culture with 9 mg/l hydrocortisone. × 40 *Figure* 5. Outgrowth area of a control culture. × 80 *Figure* 6. Higher magnification of the same culture. × 320 *Figure* 7. Outgrowth area of a 9 mg/l hydrocortisone culture. × 80 *Figure* 8. Higher magnification of the same culture. × 320

Figures 9–12. Formaldehyde-induced fluorescence in seven day cultures of sympathetic ganglia. Photographic exposure times obtained with the automatic camera are given to indicate fluorescence intensity. All figures, × 160

Figure 9. Control; exposure of negative, 160 sec. *Figure* 10. 3 mg/l hydrocortisone, 75 sec. *Figure* 11. 9 mg/l hydrocortisone, 38 sec. *Figure* 12. 9 mg/l hydrocortisone, 41 sec. Triangles indicate fluorescent fibres. Note that SIF cells in Figs. 10–12 show more intense fluorescence than those in Fig. 9 in spite of much shorter photographic exposures.

9

10

11

12

drying the specimen which allowed diffusion of catecholamines from ordinary ganglion cells. In the present study, however, this method served well for the selective demonstration of the SIF cells.

The SIF cells usually formed small and medium sized clusters (Fig. 9), which were present in varying numbers in all explants, although some individual cells were occasionally seen. The majority of the SIF cells were round or oval. However, some presumably more active cells were slightly elongated and showed a tapering of their ends. The site of SIF cell groups varied from one explant to another, although they tended to be in the peripheral regions. In all control cultures there were large areas of the explant devoid of SIF cells.

Hydrocortisone. At a concentration of 1 mg/l of hydrocortisone in the culture medium, there was a distinct increase in the intensity of the formaldehyde-induced fluorescence of the SIF cells, as indicated by shorter photographic exposures with the automatic camera and the independent visual assessment by three authors. With the filter combination BG 12 and K 530 employed, the colour of the SIF cells appeared yellow, while it was green in the controls. The vast majority of SIF cells were in the explant, mainly in the form of small- and medium-sized groups, but some SIF cells with short, broad processes were seen in the outgrowths.

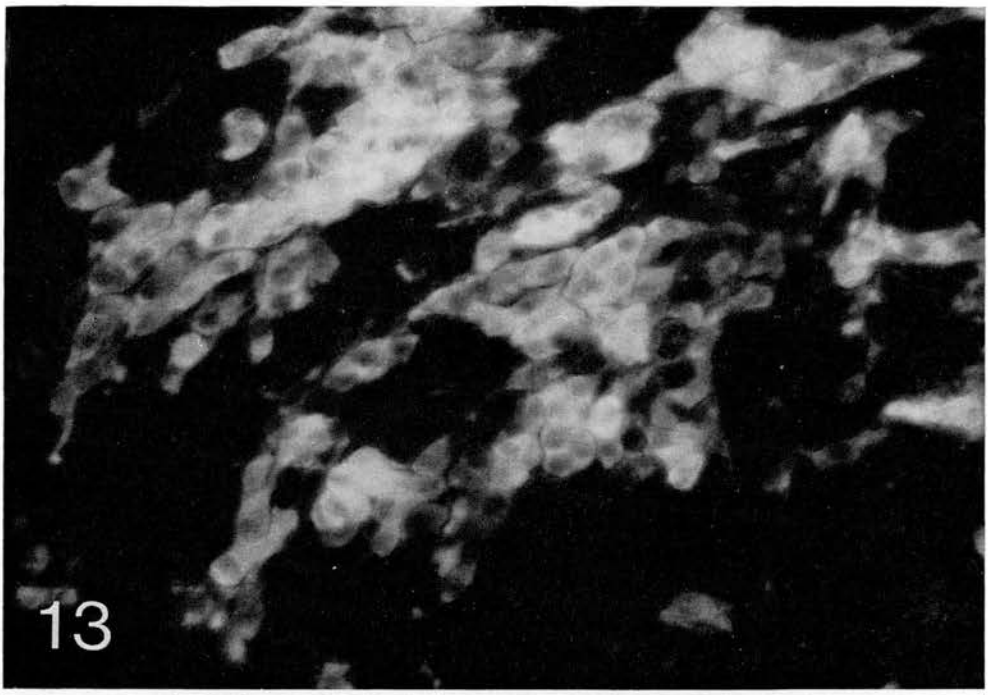
In cultures with 3 (Fig. 10) or 9 (Figs. 11 and 12) mg/l of hydrocortisone, both the number of SIF cells and their fluorescence intensities were greatly increased, the optimum photographic exposure times being much shorter than those required for the controls. Both the number of single SIF cells and that of small SIF cell groups were distinctly increased in each explant, and SIF cells were present throughout the explant (Fig. 12). Therefore, they were strikingly different from the controls, in which large areas were entirely devoid of SIF cells, although the size of the explant was about the same. In some explants, large aggregations of SIF cells were seen (Figs. 10 and 11).

Not only was the fluorescence intensity increased, but also the colour of the SIF cells changed with increasing concentration of hydrocortisone in the medium. All SIF cells were green in the control cultures and all of them yellow in the 1 mg/l cultures. In addition to yellow SIF cells, some SIF cells with a reddish tinge were seen in the 3 mg/l cultures, and such cells were more numerous in cultures with 9 mg/l hydrocortisone. These colour differences were readily distinguishable visually even with the (non-optimal) filter combination BG 12 and K 530. The yellow cells appear white and the reddish cells appear grey in Fig. 13.

While SIF cells were absent from, or few in, the outgrowth areas of the control cultures and those with 1 mg/l hydrocortisone, many SIF cells were seen in the outgrowth region of cultures with 3 mg/l, and they were even more numerous in cultures with 9 mg/l hydrocortisone. Some SIF cells had long, slender fluorescent processes. Such cells were observed both in the explant and in the outgrowth area. Some processes are indicated by triangles in Figs. 10 and 11, as well as in Fig. 14, which shows, at a higher magnification, the same area as that shown on the left in Fig. 11 (the two triangles).

Discussion

In the present study the SIF cells readily survived under tissue culture conditions. Addition of hydrocortisone to the culture medium resulted in four distinct types of



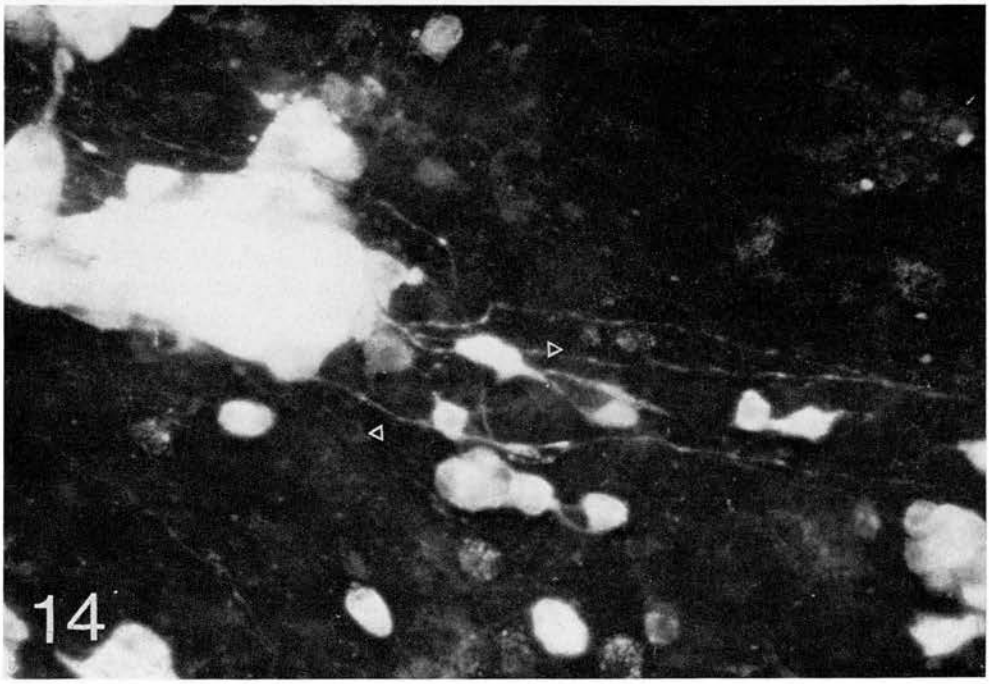


Figure 13. SIF cells in a seven day culture with 9 mg/l hydrocortisone. The light cells were yellow, the grey ones were reddish by visual observation. × 320

Figure 14. A detail of the 9 mg/l hydrocortisone culture shown in Fig. 11, the triangles in identical positions. Several beaded fluorescent fibres are visible. × 320

responses of the SIF cells: (1) marked increase in fluorescence intensity, (2) change in fluorescence colour, (3) appearance of unusually large SIF cell clusters and (4) increase in the number of single SIF cells and small SIF cell groups.

The increase in the fluorescence intensity suggests that the catecholamine concentration increased in the presence of hydrocortisone in both previously existing and potential SIF cells. An increase in the intensity of the SIF cell fluorescence was also observed in a previous paper (Eränkö & Eränkö, 1971*a*), in which the effect of injected hydrocortisone in newborn rats was studied. However, the increase in this *in vivo* study was less dramatic than in the present *in vitro* work.

The change in the colour of the SIF cells first from green to yellow and then, in some cells, to reddish with increasing concentration of hydrocortisone in the culture medium is an interesting phenomenon not observed in the *in vivo* study (Eränkö & Eränkö, 1971*a*). Such a change in the colour can be due to an increase of catecholamine concentration, a change in the catecholamine composition (dopamine, noradrenaline, adrenaline) or both (see Ritzen, 1967; Björklund *et al.*, 1970; Eränkö & Eränkö, 1971*d*). Further studies are, therefore, required to find out whether hydrocortisone causes changes in the catecholamine composition of the SIF cells. Microspectrofluorometric studies could be expected to throw further light on this problem.

An increase in the number of SIF cells may be brought about (1) by division of previously existing SIF cells, (2) by synthesis of catecholamines in already determined but not yet fluorescent SIF cells and (3) by differentiation of pluripotent cells into SIF cells, rather than ordinary nerve cells.

The presence of very large clusters of SIF cells in cultures with 9 mg/l hydrocortisone suggests formation of new SIF cells by division of SIF cells. This is a change which was not observed in the sympathetic ganglia of newborn rats injected *in vivo* with hydrocortisone (Eränkö & Eränkö, 1971*a*).

The most striking change in the cultures containing 3 or 9 mg/l hydrocortisone was the appearance of single SIF cells and small groups of SIF cells in the explants, and to a certain degree also in the outgrowth. This strongly suggests, as did the results of the *in vivo* study (Eränkö & Eränkö, 1971*a*), that hydrocortisone either has a truly inductive action resulting in the formation of SIF cells or causes an increased catecholamine synthesis in already differentiated but not yet catecholamine-containing cells (see also Lempinen, 1964).

It should be borne in mind that the increase in the number of SIF cells, as defined on the basis of the small size of the cells and their intense fluorescence only, does not necessarily mean that the new SIF cells are similar to those found in the sympathetic ganglia of normal animals. While the catecholamines are mainly concentrated in granular vesicles in the normal SIF cells found in sympathetic ganglia, it has been suggested that the SIF cells formed in newborn rats during hydrocortisone treatment do not always contain large numbers of such granular vesicles although fluorescence histochemical evidence clearly shows that high concentrations of catecholamine are stored in the cytoplasm (Eränkö & Eränkö, 1971*a*). Indeed, it seems likely that there is a continuous range of SIF cells from nerve cells unusually rich in catecholamines to the extreme chromaffin cells with large granular vesicles.

Although further studies are necessary to elucidate the mechanisms by which hydrocortisone causes its powerful action on SIF cells, the fact that dramatic changes can be

observed in cultured pieces of sympathetic ganglia indicates that this effect is a direct one and is not mediated by any system other than those present in the ganglion itself.

It is of interest that hydrocortisone causes the appearance of adrenaline in normally noradrenaline-storing extra-adrenal chromaffin cells (Eränkö *et al.*, 1966), probably because it induces activation of phenylethanolamine-N-methyl transferase (Axelrod, 1966), the enzyme which converts noradrenaline into adrenaline. Ecdysone, a steroid related to hydrocortisone, causes induction of dihydroxyphenylalanine decarboxylase in insects and an increase of glutamic decarboxylase activity in rat brain (Chaudhary *et al.*, 1969). Moreover, hydrocortisone has been shown to cause a specific synthesis of non-histone nuclear protein in rat liver (Shelton & Allfrey, 1970). It is, therefore, possible that the increase in the catecholamine content of the SIF cells observed in our study is due to hydrocortisone-induced formation of enzymes involved in catecholamine synthesis.

Acknowledgement

This study has been supported by a grant from the Sigrid Jusélius Foundation to O.E.

References

AXELROD, J. (1966). Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol. Rev.* **18,** 95–113.

BJÖRKLUND, A., CEGRELL, L., FALCK, B., RITZEN, M. & ROSENGREN, E. (1970). Dopamine-containing cells in sympathetic ganglia. *Acta physiol. scand.* **78,** 334–8.

BURNSTOCK, G., EVANS, B., GANNON, B. J., HEATH, J. W. & JAMES, V. (1971). A new method of destroying adrenergic nerves in adult animals using guanethidine. *Br. J. Pharmacol.* **43,** 295–301.

CHAMLEY, J. H., MARK, G. E., CAMPBELL, G. R. & BURNSTOCK, G. (1972*a*). Sympathetic ganglia in culture. I. Neurons. *Z. Zellforsch. mikrosk. Anat.* (submitted for publication).

CHAMLEY, J. H., MARK, G. E. & BURNSTOCK, G. (1972*b*). Sympathetic ganglia in culture. II. Accessory cells. *Z. Zellforsch. mikrosk. Anat.* (submitted for publication).

CHAUDHARY, K. D., LUPIEN, P. J. & HINSE, C. (1969). Effect of ecdysone on glutamic decarboxylase in rat brain. *Experientia* **25,** 250–1.

ERÄNKÖ, L. & ERÄNKÖ, O. (1971*a*). Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. *Acta physiol. scand.* (in press).

ERÄNKÖ, L. & ERÄNKÖ, O. (1971*b*). Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat. *Acta physiol. scand.* (in press).

ERÄNKÖ, L. & ERÄNKÖ, O. (1971*c*). Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats. *Acta pharmacol. et toxicol.* (in press).

ERÄNKÖ, O. & ERÄNKÖ, L. (1971*d*). Small intensely fluorescent, granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* **34,** 39–52.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1963). Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* **58,** 285–6.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1965*a*). Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* **63,** 511–12.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1965*b*). Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. *Acta physiol. scand.* **63,** 711–12.

ERÄNKÖ, O., LEMPINEN, M. & RÄISÄNEN, L. (1966). Adrenaline and noradrenaline in the Organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. *Acta physiol. scand.* **66,** 253–4.

GRILLO, M. A. (1966). Electron microscopy of sympathetic tissue. *Pharmac. Rev.* **18,** 387–99.

HANKS, J. H. & WALLACE, R. E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. exp. Biol.* (NY). **71,** 196–200.

LEMPINEN, M. (1964). Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. *Acta physiol. scand.* **62,** Suppl. 231.

MATTHEWS, M. R. & RAISMAN, G. (1969). The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. *J. Anat.* **105,** 255–82.

NORBERG, K.-A. & HAMBERGER, B. (1964). The sympathetic adrenergic neuron. *Acta physiol. scand.* **63,** Suppl. 238.

RITZEN, M. (1967). Cytochemical Identification and Quantitation of Biogenic Monoamines. Thesis, Stockholm, P.A. Norstedt & Söner, pp. 63.

ROSE, G. C. (1954). A separable and multipurpose tissue culture chamber. *Tex. Rep. Biol. Med.* **12,** 1074–83.

SALK, E. S., YOUNGNER, J. S. & WARD, E. N. (1954). Use of color change of phenol red as the indicator in titrating poliomyelitis virus or its antibody in a tissue culture system. Appendix. Method of preparing medium 199. *Amer. J. Hyg.* **60,** 214–30.

SHELTON, K. R. & ALFREY, V. G. (1970). Selective synthesis of a nuclear acidic protein in liver cells stimulated by cortisol. *Nature, Lond.* **228,** 132–4.

VAN ORDEN, L. S., BURKE, J. P., GEYER, M. & LODEON, F. V. (1970). Localization of depletion-sensitive and depletion-resistant norepinephrine storage sites in autonomic ganglia. *J. Pharmacol. exp. Ther.* **174,** 56–71.

WILLIAMS, T. H. & PALAY, S. L. (1969). Ultrastructure of the small neurons in the superior cervical ganglion. *Brain Res.* **15,** 17–34.

Histochemical Journal, 4 (1972), 213–224

*Light and electron microscopic histochemical evidence of granular and non-granular storage of catecholamines in the sympathetic ganglion of the rat**

OLAVI ERÄNKÖ

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received 7 April 1972

Synopsis. Superior cervical ganglia of adult rats were studied for light microscopy with the formaldehyde-induced catecholamine fluorescence technique and by electron microscopy after fixation in 3% potassium permanganate in phosphate-buffered Krebs-Ringer solution.

In ganglia frozen-dried for a week at −45°C or below, the nerve cells showed a diffuse blue catecholamine fluorescence throughout the perikaryon and a more intense fluorescence in granular structures predominantly in the periphery of the cytoplasm. All the nerve cells exhibited the same blue fluorescence, although there was a great variation in the fluorescence intensity between individual cells. Clusters of small cells exhibited a very intense catecholamine fluorescence. In specimens frozen-dried at temperatures higher than −35°C or for less than a week, no granular fluorescence was observed in the perikarya or nerve cell processes.

Clusters of small granular vesicles, 30–50 nm in diameter, were observed by electron microscopy in the peripheral cytoplasm of most nerve cells. Some nerve cells showed small granular vesicles scattered throughout the perikaryon and others were entirely devoid of such vesicles. A few granular vesicles were seen in the Golgi region of most cells. Large granular vesicles, about 100 nm in diameter, were seen in many cells but their number was much smaller than that of the small granular vesicles. Small cells containing numerous granular vesicles of even larger size, about 150 nm in diameter, were also observed. They corresponded to the small very intensely fluorescent cells seen by fluorescence microscopy.

It is concluded, firstly, that the fluorescent granules correspond to the clusters of small granular vesicles demonstrable by electron microscopy. Since most nerve cells showed small granular vesicles only in the periphery of the cytoplasm but a diffuse fluorescence throughout the cytoplasm, it is concluded, secondly, that a large proportion of the cytoplasmic catecholamines is stored in a non-granular pool, presumably in the endoplasmic reticulum.

* This study was carried out in the University of Helsinki. The manuscript was completed in the University of Melbourne, where the author was a Senior Research Fellow from September 1971 to August 1972.

Introduction

The mode of storage of catecholamines in the cytoplasm of the sympathetic ganglion cells has been extensively studied using a variety of biochemical, histochemical and morphological methods. However, the results obtained are markedly controversial.

Using the formaldehyde-induced fluorescence method (Eränkö, 1955, 1967; Falck, 1962) for the histochemical demonstration of noradrenaline, Eränkö & Härkönen (1963) reported a diffuse fluorescence in the cytoplasm of all nerve cells of the superior cervical ganglion of the rat and brilliantly fluorescent small granules in the cytoplasm of many cells. In a similar study, Norberg & Hamberger (1964) observed only the diffuse fluorescence in the cytoplasm and considered the granular appearance an artifact. Of two recent fluorescence microscope studies on the same rat ganglion, one (Jacobowitz, 1970) reported a diffuse catecholamine fluorescence, the other (Van Orden *et al.*, 1970*a*) confirmed the presence of fluorescent granules in the cytoplasm of the sympathetic nerve cells.

Following the preparation of a catecholamine-rich granular fraction from homogenates of adrenergic nerves by Euler & Hillarp (1956) it has been generally accepted that catecholamines are stored in granules in the peripheral sympathetic nerves (see review by Geffen & Livett, 1971). However, chemical fractionation studies on sympathetic ganglia have not provided similar clear-cut results. Thus, Fischer & Snyder (1965) concluded that both endogenous and exogenous catecholamines in the superior cervical ganglion of the rat were essentially confined to the supernatant fraction, and this view was shared by Axelrod (1970). On the other hand, Philippu *et al.* (1967) have reported separation of a granular fraction rich in noradrenaline from the stellate ganglion of the cow, although they did not mention how much of the original noradrenaline content was recovered in the granular fraction.

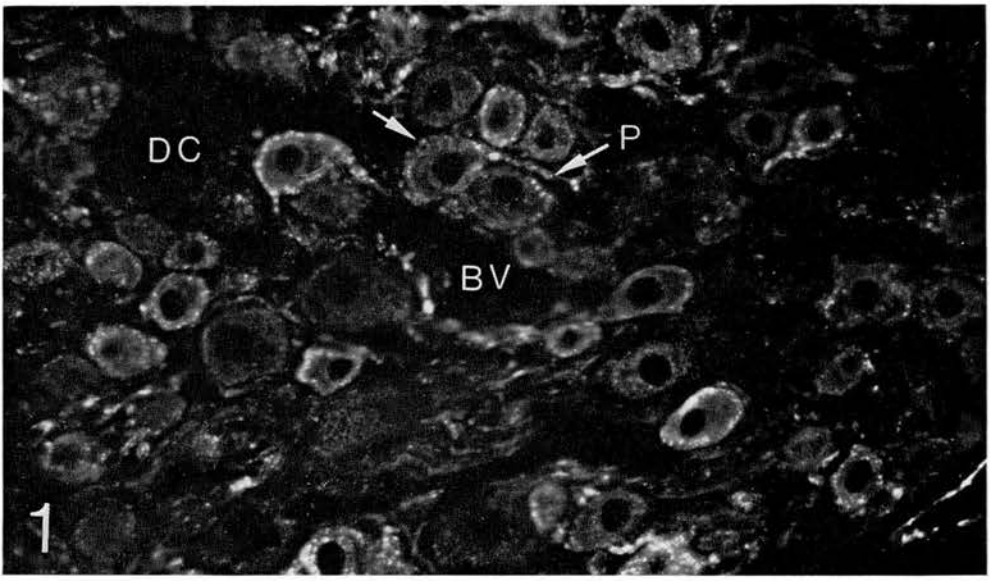
There is strong electron microscopic evidence that catecholamines are stored in small granular vesicles about 50 nm in diameter in the peripheral adrenergic nerve terminals (De Robertis & Pellegrino de Iraldi, 1961; Richardson, 1964, 1966; Grillo, 1966; Machado, 1971; Pellegrino de Iraldi *et al.*, 1971). Using Richardson's (1964, 1966) permanganate fixation method, which is the most sensitive ultrastructural histochemical method for this purpose, such granular vesicles have recently been demonstrated also in the cytoplasm of the nerve cells in sympathetic ganglia (Hökfelt, 1969, 1971; Van Orden *et al.*, 1970*a*; Hervonen, 1971; Eränkö & Eränkö, 1972). From morphological considerations it has, indeed, been claimed that neuronal catecholamines are exclusively stored in the 'storage' granules (Dahlström, 1967) seen in electron microscopy.

In the present study, the problem of noradrenaline storage in the nerve cells of the sympathetic ganglion has been approached by comparing the distribution of the formaldehyde-induced catecholamine fluorescence with that of the small granular vesicles demonstrable by electron microscopy after fixation with potassium permanganate. Evidence is presented for the existence of both the granular and non-granular modes of storage of noradrenaline in the cytoplasm of sympathetic nerve cells.

Materials and methods

Sympathetic ganglia

Adult male albino rats of the colony in the Department of Anatomy, descendants of rats of the Sprague-Dawley strain, were used. The animals were briefly anaesthetized with



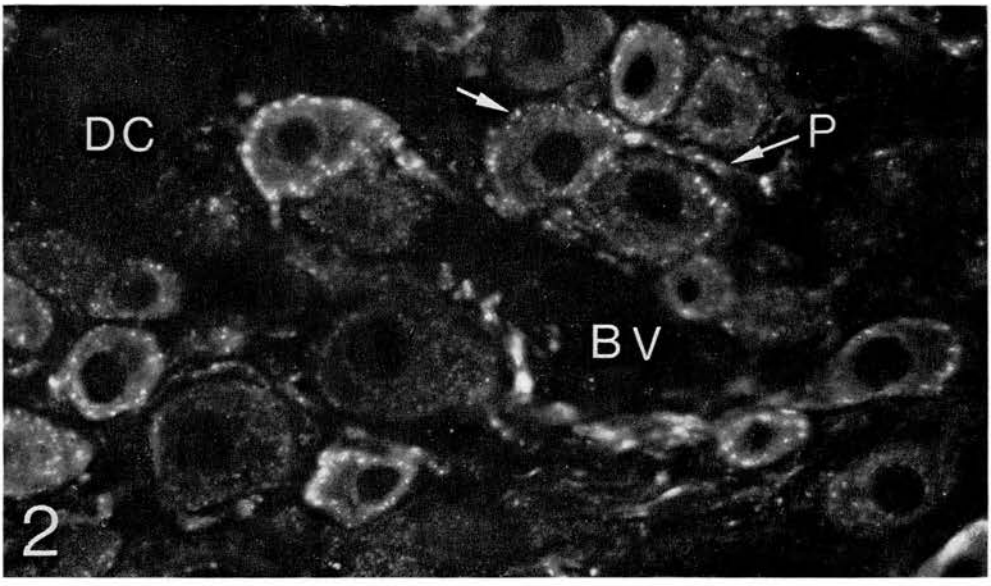


Figure 1. Formaldehyde-induced catecholamine fluorescence in the superior cervical ganglion of the rat. General view showing the variation in the fluorescence intensity between the individual cells, one of which (DC, dark cell) shows a very weak fluorescence. Arrows indicate a nerve cell body with peripheral fluorescent granules and its process (P). BV, blood vessel. × 320

Figure 2. Higher magnification of the same field. The arrows and abbreviations are the same as in Fig. 1. The peripheral fluorescent granules in the cytoplasm and in the process (P) are clearly visible; these granules exhibited a specific blue catecholamine fluorescence, as did the even fluorescence throughout the cytoplasm. × 510

ether and killed immediately after they had become immobile by cutting with sharp scissors the vertebral column, spinal cord and aorta at the level of the heart.

The superior cervical ganglia were removed within 1 min of killing. Handling of the ganglia themselves was avoided by holding with forceps the preganglionic nerve trunk only.

Fluorescence microscope methods

Whole ganglia were placed on thin copper netting and immersed on it in propane cooled to −190°C with liquid nitrogen. The pieces were then freeze-dried for 1–7 days at −30, −40, −45 or −50°C in a desiccator containing phosphorus pentoxide above the tissue holder and evacuated with a combination of a mechanical and a diffusion pump. After the selected drying period, the desiccator was warmed up to +50°C, the vacuum was broken and the ganglia were transferred to glass jars containing paraformaldehyde powder equilibriated with air of 60% relative humidity. The jar was kept for 30 min in an incubator at 50°C and thereafter for 60 min at 80°C.

After exposure to formaldehyde vapour, the ganglia were embedded in paraffin wax or in Epon-Araldite mixture. Sections were cut at 3–10 μm from specimens embedded in paraffin wax. Resin-embedded ganglia were cut dry with the LKB Pyramitome at 1–5 μm. Sections were embedded in Entellan or liquid paraffin and examined by fluorescence microscopy using a Leitz Ortholux microscope with a HBO 200 mercury lamp and the following filters: BG 38, BG 3, TAL 405, K 460. Details of the method have been described in previous papers (Eränkö, 1967; Eränkö & Eränkö, 1971).

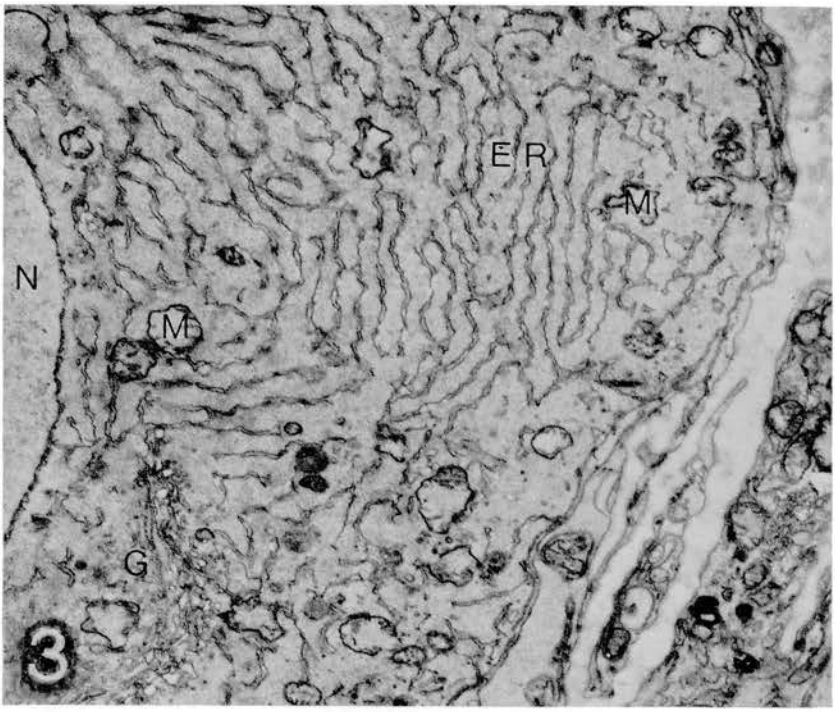
Electron microscopy methods

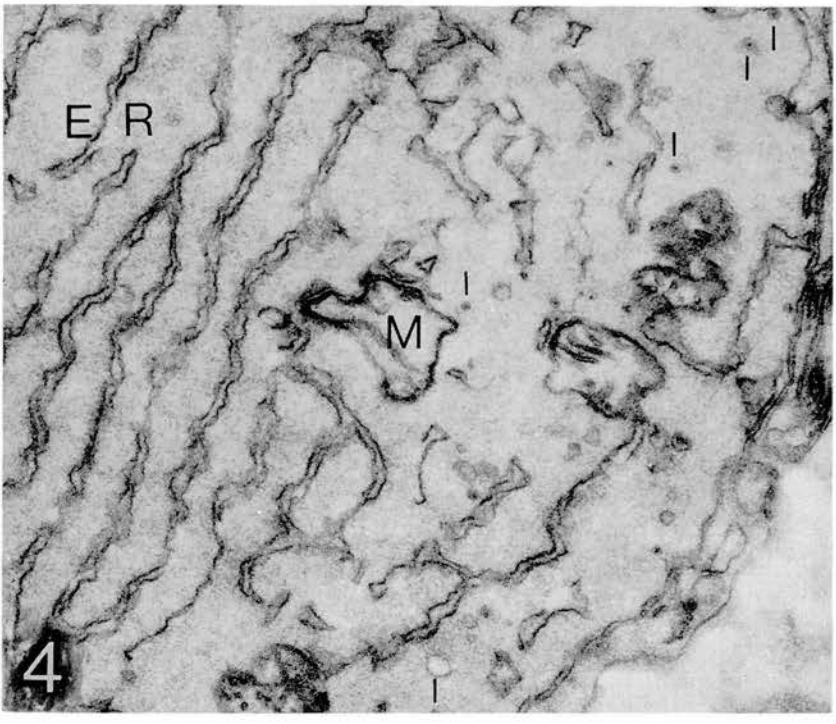
Immediately after removal, the ganglia were placed in a drop of fixative on a glass plate kept at 0°C over crushed ice. The ganglion was then cut with sharp eye-scissors into small pieces in the fixative. These were subsequently transferred into a larger volume of fixative in weighing bottles, which were also kept in crushed ice.

The main fixative consisted of 3% potassium permanganate (Richardson, 1966) in phosphate-buffered Krebs-Ringer solution (Eränkö *et al.*, 1967) adjusted to pH 7.0. This fixation fluid was essentially similar to that used by Hökfelt (1968) and Van Orden *et al.* (1970*a*). After fixation for 45 min at 0°C, the tissue pieces were rinsed in three 10 min changes of the Krebs-Ringer solution at 0°C. They were then dehydrated and embedded in a mixture of Araldite 502 and Epon 812 as described previously (Eränkö & Eränkö, 1971.) Thin sections were cut with a LKB Ultrotome and placed on copper grids covered with a Formvar film. The sections were stained on grids with lead citrate. AEI 801 and Philips EM 300 electron microscopes were used at 40 or 50 kV.

Figure 3. Low-power electron micrograph of a typical sympathetic ganglion cell fixed in 3% potassium permanganate in phosphate-buffered Krebs-Ringer solution. Note the well-developed endoplasmic reticulum (ER), most of which is granular, although the ribosomes do not show with this fixation. The nucleus (N) is on the left. M, mitochondria; G, the Golgi apparatus. × 6000

Figure 4. A detail of the upper right corner of Fig. 3, with the markings ER and M in the same positions. Several small granular vesicles near the outer rim of the cytoplasm on the right, some of which have been marked with vertical lines. × 17,000


E R
M
N
M
G
3


E R
I
I
I
I
M
I
4

Some ganglia were fixed at 0°C for 1 hr in 3.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, rinsed overnight in the buffer and postfixed at 0°C for 1 hr in 2% osmium tetroxide in distilled water. After three 10 min rinses in distilled water, the tissues were dehydrated and mounted as described above.

Results

Freeze-drying

Good, reproduceable results were obtained after freeze-drying for 7 days at −45 or −50°C. Ganglia dried at −30°C always showed signs of diffusion, while variable results were obtained after drying at −35 or −40°C for 1–7 days. Even at −45 or −50°C, diffusion was observed if drying times shorter than a week were employed. These temperatures refer to those in the freezer; it is likely that the actual tissue temperatures were somewhat lower. The following description is based on results obtained after freeze-drying for 7 days at −45 or −50°C.

Catecholamine fluorescence

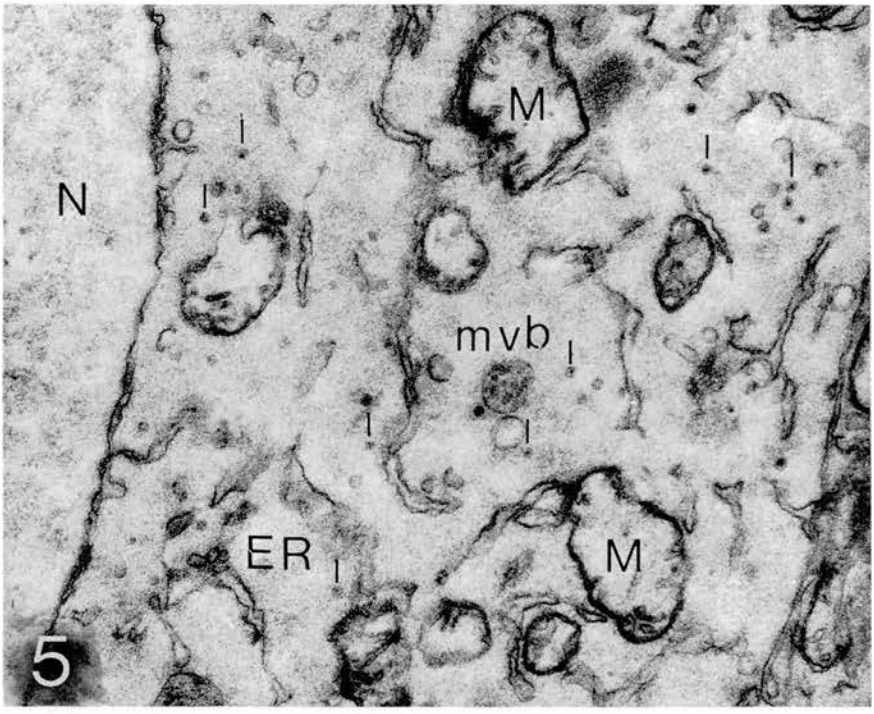
The distribution of the formaldehyde-induced fluorescence in a 4 μm thick section of the superior cervical ganglion of a rat is illustrated in Fig. 1. The intense granular fluorescence in the periphery of the nerve cell bodies and in the numerous nerve fibres (P) between them is shown more clearly in Fig. 2. It was a characteristic observation in all sections dried under optimum conditions. Even in such sections, however, an even fluorescence was observed all through the nerve cell cytoplasm from the nuclear membrane to the cell periphery. Such specific blue fluorescence was especially clear in thicker sections cut through the centre of the fluorescent nerve cell body, in which the non-fluorescent nucleus was seen in striking contrast. The diffuse component of the fluorescence faded rapidly in thin sections, and the particulate fluorescence, which was more resistant, became even more clearly visible. In sections exposed for long periods to u.v. light, even this peripheral catecholamine fluorescence disappeared and only a non-specific fluorescence resistant to u.v. light was seen in the cytoplasm. Such fluorescence was not formaldehyde-induced, that is it was not due to catecholamines, as was shown by examination of control sections not exposed to formaldehyde. In such sections, numerous cytoplasmic 'granules' (mitochondria or lysosomes ?) exhibiting an orange or red-orange fluorescence were seen in the nerve cell bodies and were distributed throughout the perikaryon except for a narrow rim in the periphery of the cell, which was the most typical site of the catecholamine 'granules' in formaldehyde-exposed tissues.

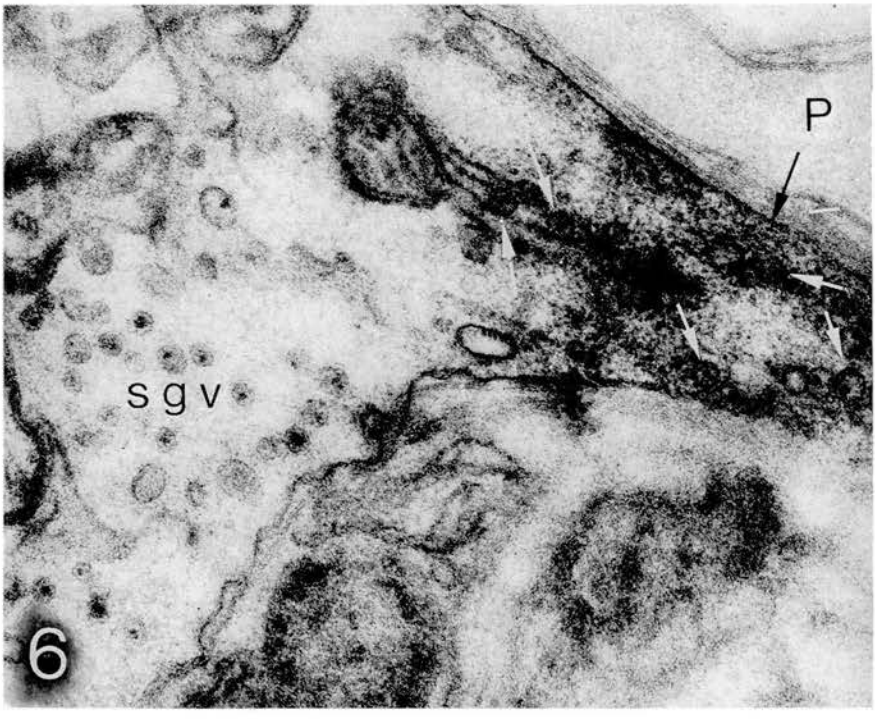
Small intensely fluorescent (SIF) cells were clearly visible in all specimens, without regard to the drying temperature or the length of the drying period. These cells have

---

Figure 5. A cell showing small granular vesicles (marked with vertical lines) in all parts of the cytoplasm. The endoplasmic reticulum (ER) is scarce. A multivesicular body (mvb) can be seen in the centre. M, mitochondria; N, nucleus. Technique as in Figs. 3 and 4. × 17,000

Figure 6. Part of a nerve cell like that marked with arrows in Figs. 1 and 2. A cluster of small granular vesicles (sgv) is seen in the cytoplasm and the process (P) emerging from it also contains numerous vesicles of the same type (some marked with arrows). Permanganate fixation. × 34,500


N
M
mvb
ER
M
5


P
sgv
6

been studied in detail in a separate paper (Eränkö & Eränkö, 1971). Here it is sufficient to note that they served as good indicators of diffusion because of their high catecholamine content. In poorly dried specimens the outlines of the SIF cells were indistinct.

Electron microscopy

Fig. 3 is a low-power electron micrograph of a typical nerve cell of rat superior cervical ganglion. It illustrates the general features observed in the majority of nerve cells in this ganglion: a vesicular nucleus with numerous fenestrations, a well-developed endoplasmic reticulum, a well-developed Golgi apparatus, and numerous mitochondria. A narrow layer of Schwann cell cytoplasm surrounded the nerve cell.

Small granular vesicles, barely visible at this magnification, were detected in the periphery of the cytoplasm of this cell, the large central area being devoid of them. Some vesicles are indicated by vertical lines in Fig. 4, which was taken at a higher magnification of the same cell as that in Fig. 3. Large clusters of small granular vesicles were often seen near the origin of and inside such nervous processes which were in open cytoplasmic contact with the perikaryon (P in Fig. 6). A cluster in the periphery of another nerve cell is illustrated in Fig. 7, in which the membranous vesicle surrounding the granule is clearly visible. This membrane was triple layered. The diameter of the small granular vesicles was 30–50 nm. Nerve fibres enclosed in the cytoplasm of Schwann cells in the space between the nerve cell bodies also showed clusters of small granular vesicles. Similar vesicles were observed near the Golgi apparatus as well (Fig. 8), although they were there very much less numerous than in the periphery.

Occasional large granular vesicles, about 100 nm in diameter, were observed in the Golgi region, in the periphery of the cytoplasm, in the cell processes and in the nerve fibres surrounded by Schwann cells. However, these granular vesicles were few.

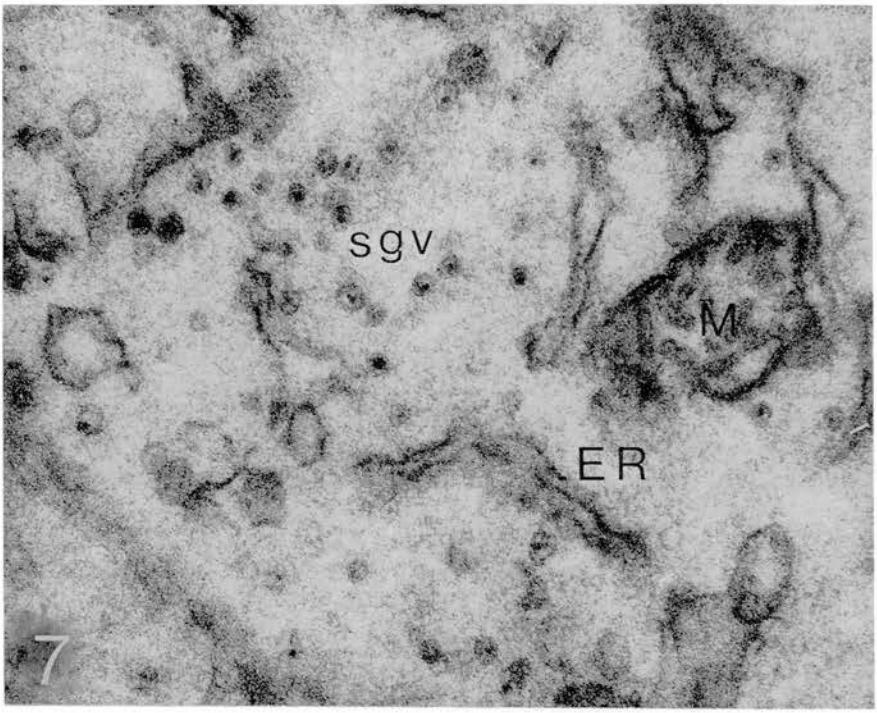
Thus, the most common type of nerve cell contained numerous small granular vesicles within a narrow peripheral rim, a few of them near the Golgi apparatus and the nucleus but none in the large space between the close perinuclear and the extreme peripheral regions of the cell occupied by the endoplasmic reticulum. However, a second type of nerve cell was also observed in which the small granular vesicles were evenly scattered in the perikaryon (Fig. 5). These cells distinctly differed from small cells which contained scores of large granular vesicles, 100 nm or more in diameter, and probably corresponded to the small intensely fluorescent cells (see Eränkö & Eränkö, 1971).

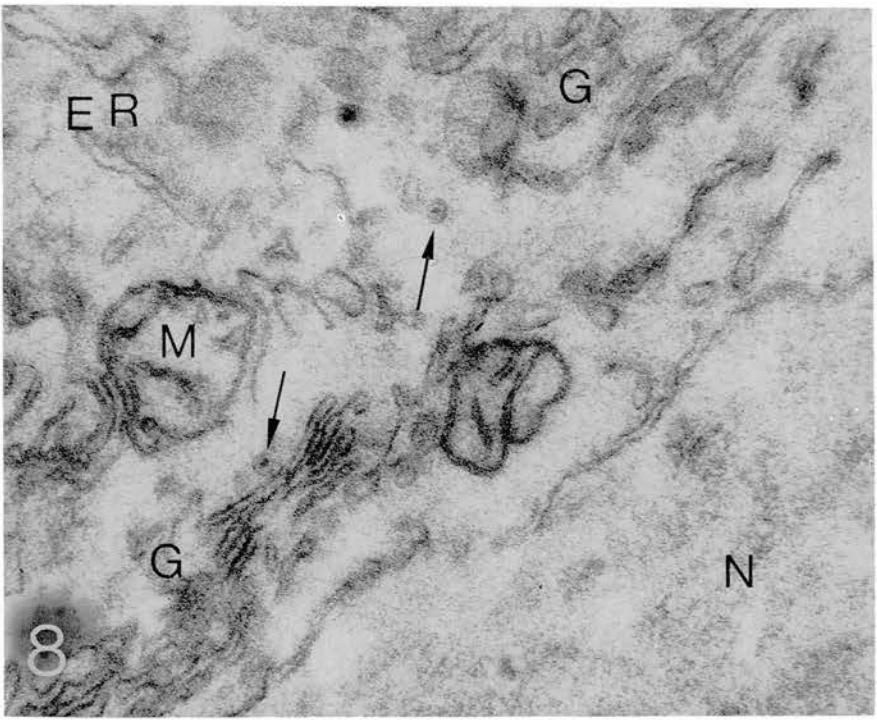
Finally, nerve cell profiles of a third type were observed in which no granular vesicles were found at all in spite of careful scanning of the whole perikaryon at a high magnification.

No synaptic terminals filled with small granular vesicles were seen apposed on the surface membrane of the nerve cells as was postulated by Norberg & Hamberger (1964), neither were there short processes with granular vesicles such as those described in the inferior mesenteric ganglion of the cat by Elfvin (1971).

Figure 7. Cluster of small granular vesicles (sgv) in the cytoplasm of a nerve cell. ER, endoplasmic reticulum; M, mitochondrion. Permanganate fixation. × 34,500

Figure 8. Perinuclear region of a nerve cell showing two small granular vesicles (marked with arrows) near the Golgi apparatus (G). ER, endoplasmic reticulum; N, nucleus. Permanganate fixation. × 34,500


sgv
M
ER
7


ER
G
M
G
N
8

Discussion

Fluorescence microscopic observations of the present study confirmed the presence of both the diffuse and the granular component of histochemically demonstrable catecholamines in the cytoplasm of sympathetic nerve cells previously reported by Eränkö & Härkönen (1963) and Van Orden *et al.* (1970*a*). Moreover, it was shown that the intensely fluorescent cytoplasmic granules can only be adequately demonstrated if the freeze-drying temperature is low enough and the drying time sufficiently long. Otherwise diffusion of catecholamines occurs from the cytoplasmic granules during drying and a fairly diffuse fluorescence is seen in the cytoplasm of the nerve cells. It is likely that such diffusion explains why Norberg & Hamberger (1964) and Jacobowitz (1970) failed to observe intensely fluorescent granules in the periphery of the nerve cells.

Further support to the view that the cytoplasmic granules with an intense fluorescence are true neuronal structures is given by the electron microscopic observation of numerous small granular vesicles in the periphery of the nerve cell perikaryon and in the neuronal processes between the nerve cell bodies, both typical sites of intense granular fluorescence. Clusters of small granular vesicles have been previously reported in the peripheral areas of the nerve cell bodies of the superior cervical ganglion of the rat by Hökfelt (1969) and Van Orden *et al.* (1970*a*). Van Orden *et al.* (1970*a*) correlated light and electron microscopic observations and concluded that the granular appearance on fluorescence microscopy is not an artifact, but that each fluorescent granule represents a cluster of noradrenaline-storing granular vesicles. The results of the present study are in full agreement with this conclusion.

It was also shown in the present study that catecholamine accumulations represented by fluorescent granules in light microscopy were not synaptic knobs with small granular vesicles in contact with the nerve cells, as was proposed by Norberg & Hamberger (1964) as a possible explanation to the granular appearances.

While it seems quite clear, then, that granular catecholamine fluorescence can always be found in sites containing small granular vesicles, the question arises whether the reverse is true, that is, whether small granular vesicles represent the only mode of catecholamine storage in the neuron. On the basis of simultaneous changes in the noradrenaline concentration and the number of small granular vesicles, these have indeed been proposed as the only site of noradrenaline storage (Dahlström, 1967). However, although granular fractions have been separated from homogenates of the stellate ganglion of the cow (Philippu *et al.*, 1967), previous studies on the superior cervical ganglion of the rat have revealed that almost all catecholamines are recovered in the supernatant (Fischer & Snyder, 1965; Axelrod, 1970). This can, of course, be explained by assuming that neuronal catecholamine-storing vesicles release their catecholamines during homogenization, but the relative stability of the granular fraction separated by Philippu *et al.* (1967) renders such a view unlikely.

An alternative explanation is that a large part of the total noradrenaline content of the sympathetic nerve cells is stored outside the granular vesicles in a 'diffuse' pool. The presence of such a pool has been postulated previously in the early chemical fractionation study by Euler & Hillarp (1956) and in the fluorescence histochemical paper by Eränkö & Härkönen (1963). The observations of the present study strongly support such a non-granular mode of storage of noradrenaline: a specific formaldehyde-induced fluorescence

was found evenly distributed in the whole cytoplasm of almost all nerve cells, in addition to the granular fluorescence in the periphery of the cytoplasm, while on electron microscopic examination most nerve cells failed to show any granular vesicles in the region of such diffuse fluorescence. Therefore, it seems very likely that a large proportion of catecholamines is contained in an extragranular compartment in the perikarya of normal sympathetic ganglion cells.

This may also apply to peripheral adrenergic nerves, although to a less extent. Van Orden *et al.* (1970*b*) observed a loss of noradrenaline from the adrenergic nerves of the vas deferens of the rat after treatment with α-methyl-*p*-tyrosine, although no change was found in the number or appearance of the small granular vesicles. This observation strongly suggests that there is a non-granular pool of catecholamines in the peripheral portions of at least some adrenergic neurons. Such a pool explains the presence of relatively large amounts of catecholamines in the supernatant of centrifuged homogenates of adrenergically innervated organs (Iversen, 1967; Geffen & Livett, 1971).

Although considerable amounts of noradrenaline may be stored outside the pool formed by the small granular vesicles in the perikaryon of sympathetic nerve cells, as is suggested by the relatively large cytoplasmic area of the diffuse fluorescence component, this does not necessarily mean that all noradrenaline in this non-granular pool is simply dissolved and freely diffusible in the cytoplasm of the living cell. It seems more likely that a considerable part of total catecholamines is bound within membranous structures, although more loosely than catecholamines in the granular vesicles. If catecholamines were inside the membranes of the endoplasmic reticulum, they would be intramembranous, although non-granular, and thus protected from monoamine oxidase. Membranes of the endoplasmic reticulum were indeed plentiful in those areas of the perikaryon in which the diffuse cytoplasmic fluorescence was observed. It seems also possible that axoplasmic transport of noradrenaline may occur not only in the small granular vesicles, known to move in the axon, but also in a non-particulate form in the microtubules, which play an important role in the axonal transport (Mayor *et al.*, 1970).

Acknowledgements

The author is very grateful to Miss Laila Hänninen for embedding, cutting and staining the electron microscopic preparations with great skill. Mrs Tuula Stjernvall cut the light microscopic sections, which is acknowledged with many thanks.

Thanks are due to the Sigrid Jusélius Foundation, who supported the present work with a grant.

References

AXELROD, J. (1970). Personal communication to Van Orden *et al.* (1970*a*). *loc. cit.*

DAHLSTRÖM, A. (1967). The intraneuronal distribution of noradrenaline and the transport and life-span of amine storage granules in the sympathetic adrenergic neuron. *Naynyn-Schmiedebergs Arch. Pharmakol. u. exp. Path.* **257**, 93–115.

DE ROBERTIS, E. & PELLEGRINO DE IRALDI, A. (1961). Plurivesicular secretory processes and nerve endings in the pineal gland. *J. biophys. biochem. Cytol.* **10**, 361–72.

ELFVIN, L. F. (1971). Ultrastructural studies on the synaptology of the inferior mesenteric ganglion of the cat. *J. ultrastruc. Res.* **37**, 411–48.

ERÄNKÖ, L. & ERÄNKÖ, O. (1972). Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat. *Acta physiol. scand.* **84,** 115–24.

ERÄNKÖ, O. (1955). Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. *Acta endocrinol.* **18,** 174–9.

ERÄNKÖ, O. (1967). The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. R. microsc. Soc.* **87,** 259–76.

ERÄNKÖ, O. & ERÄNKÖ, L. (1971). Small, intensely fluorescent, granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Res.* **34,** 39–52.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1963). Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* **58,** 285–6.

ERÄNKÖ, O., KOELLE, G. B. & RÄISÄNEN, L. (1967). A thiocholine-lead ferrocyanide method for acetylcholinesterase. *J. Histochem. Cytochem.* **15,** 674–79.

EULER, U. S. von & HILLARP, N. Å. (1956). Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Nature, Lond.* **177,** 44–5.

FALCK, B. (1962). Observations on the possibilities of the cellular localization of monoamines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.* **10,** 348–54.

FISCHER, J. E. & SNYDER, S. (1965). Disposition of norepinephrine – ^{3}H in sympathetic ganglia. *J. Pharmacol. exp. Ther.* **150,** 190–5.

GEFFEN, L. B. & LIVETT, B. G. (1971). Synaptic vesicles in sympathetic neurons. *Physiol. Rev.* **51,** 98–157.

GRILLO, M. A. (1966). Electron microscopy of sympathetic tissues. *Pharmacol. Rev.* **18,** 387–99.

HERVONEN, A. (1971). Development of catecholamine-storing cells in human fetal paraganglia and adrenal medulla. *Acta physiol. scand.* **63,** Suppl. 368. 1–94.

HÖKFELT, T. (1968). In vitro studies on central and peripheral monoamine neurons at the ultrastructural level. *Z. Zellforsch.* **91,** 1–74.

HÖKFELT, T. (1969). Distribution of noradrenaline storing particles in peripheral adrenergic neurons as revealed by electron microscopy. *Acta physiol. scand.* **76,** 427–40.

HÖKFELT, T. (1971). Ultrastructural localization of intraneuronal monoamines. Some aspects on methodology. *Prog. Brain Res.* **34,** 213–22.

IVERSEN, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves.* pp. 253. London, Cambridge University Press.

JACOBOWITZ, D. (1970). Catecholamine fluorescence studies of adrenergic neurons and chromaffin cells in sympathetic ganglia. *Fedn Proc.* **29,** 1929–44.

MACHADO, A. B. M. (1971). Electron microscopy of developing sympathetic fibres in the rat pineal body. The formation of granular vesicles. *Prog. Brain Res.* **34,** 171–85.

MAYOR, D., BANKS, P., TOMLINSON, D. R. & GRIGAS, R. (1971). Noradrenaline transport in sympathetic nerves. *Prog. Brain Res.* **34,** 489–98.

NORBERG, K. A. & HAMBERGER, B. (1964). The sympathetic adrenergic neuron. *Acta physiol. scand.* **63,** Suppl. 238. 1–42.

PELLEGRINO DE IRALDI, A., GUEUDET, R. & SUBURO, A. M. (1971). Differentiation between 5-hydroxytryptamine and catecholamines in synaptic vesicles. *Prog. Brain Res.* **34,** 161–70.

PHILIPPU, A., PFEIFFER, R., SCHÜMANN, H. J. & LICKEFELD, K. (1967). Eigenschaften der Noradrenaline speichernden Granula des sympathischen Ganglion stellatum. *Naynyn-Schmiedebergs Arch. Pharmakol. u. exp. Path.* **258,** 251–65.

RICHARDSON, K. C. (1964). The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles. *Amer. J. Anat.* **114,** 173–206.

RICHARDSON, K. C. (1966). Electron microscopic identification of autonomic nerve endings. *Nature, Lond.* **210,** 756.

VAN ORDEN, L. S. III, BURKE, J. P., GEYER, M, & LODOEN, F. V. (1970*a*). Localization of depletion-sensitive and depletion-resistant norepinephrine storage sites in autonomic ganglia. *J. Pharmacol. exp. Ther.* **174,** 56–71.

VAN ORDEN, L. S. III, SCHAEFER, J. M., BURKE, J. P. & LODOEN, F. V. (1970*b*). Differentiation of norepinephrine storage compartments in peripheral adrenergic nerves. *J. Pharmacol. exp. Ther.* **174,** 357–68.

Reprinted from
Brain Research
Elsevier Publishing Company, Amsterdam
Printed in the Netherlands

LACK OF TOXIC EFFECT OF GUANETHIDINE ON NERVE CELLS AND SMALL INTENSELY FLUORESCENT CELLS IN CULTURES OF SYMPATHETIC GANGLIA OF NEWBORN RATS

LIISA ERÄNKÖ*, CARYL HILL**, OLAVI ERÄNKÖ*** AND GEOFFREY BURNSTOCK

Department of Zoology, University of Melbourne, Parkville, Victoria 3052 (Australia)

(Accepted February 22nd, 1972)

INTRODUCTION

Chronic injections of guanethidine have recently been shown to produce degenerative changes in adrenergic neurones[3,7,9,14,16–19]. In contrast, the small intensely fluorescent (SIF) cells, first described in the superior cervical ganglion of the rat[12,13] and subsequently studied in many sympathetic ganglia of several species[10,23], are not affected by treatment with guanethidine in adult rats[3,7]. Furthermore, the SIF cells are substantially increased in number if guanethidine is injected into newborn animals[7].

The mechanism of these actions of guanethidine is not clearly understood, although a direct toxic effect on the nerve cells has been proposed[19]. In the present experiments this question is investigated by examining the prolonged action of guanethidine on tissue cultures of sympathetic neurones and SIF cells.

MATERIALS AND METHODS

Tissue culture

Sympathetic ganglia were obtained from newborn rats of the Sprague–Dawley strain. Both the superior cervical and the thoracic chain ganglia were cultured.

The method of tissue culture developed in this laboratory is described in detail elsewhere[5]. After aseptic dissection, adherent fat and connective tissue were carefully removed with watchmakers forceps and, in the case of the thoracic chain, the ganglia

---

* Sunshine Foundation and Rowden White Overseas Research Fellow in the University of Melbourne, September, 1971–August, 1972. Permanent address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki 00170, Finland.

** Holder of a Commonwealth of Australia Postgraduate Award.

*** University of Melbourne Senior Research Fellow, September, 1971–August, 1972. Permanent address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki 00170, Finland.

were isolated by cutting away the interganglionic connectives. Where necessary, the ganglia were cut into small pieces. The pieces were kept moist at all times with Hanks balanced salt solution[15] with 10% (v/v) foetal calf serum, 100,000 I.U./l penicillin sodium G, 100 mg/l streptomycin sulphate and 0.2 g/l phenol red as a pH indicator.

All ganglionic material was placed on collagen-coated coverslips under strips of dialysing cellophane (Visking Co., Division of Union Carbide, Chicago, Ill., size 27/32, average pore radius 2.4 nm) in modified Rose[24] chambers. Medium 199 (ref. 25) supplemented with 20% (v/v) foetal calf serum, 100,000 I.U./l penicillin sodium G, 50 I.U./l insulin and an extra 5 g/l glucose, was used in control cultures. Guanethidine sulphate was dissolved in Hanks balanced salt solution in a concentration of 1 g/l, sterilised by filtration through a 200 nm Millipore filter and diluted with the above nutrient medium to give final concentrations of 1, 3, 9 or 36 mg/l. The medium was changed daily in all chambers and the cultures were incubated at 37 °C in an atmosphere supplied with 5% carbon dioxide in air bubbled through water. Growth of the cultures was examined by phase contrast and dark ground microscopy every day and recorded by photomicrography every other day (Zeiss Standard RA microscope, with Zeiss Ikon automatic camera).

The following experiments were carried out:

Experiment 1. Cultures of the superior cervical ganglion with 0, 1, 3 or 9 mg/l of guanethidine sulphate for a period of 6 days.

Experiment 2. Cultures of the thoracic chain ganglia with 0, 1, 3 or 9 mg/l of guanethidine sulphate for a period of 8 days.

Experiment 3. Cultures of the thoracic chain ganglia with 0, 1, 3 or 9 mg/l of guanethidine sulphate for a period of 6 days.

Experiment 4. Cultures of the thoracic chain ganglia with 0, 1, 3 or 9 mg/l of guanethidine sulphate for a period of 6 days followed by a further 7 days during which control medium was supplied to all cultures.

Experiment 5. Cultures of the thoracic chain ganglia with 36 mg/l of guanethidine sulphate for a period of 7 days.

Histochemical demonstration of catecholamines

At the end of each culture period, both control and treated cultures were prepared for fluorescence histochemistry. The chambers were dismantled and the tissue, growing on the bottom coverslip, washed gently with serum-free Hanks balanced salt solution before being dried over phosphorus pentoxide overnight in a vacuum desiccator. The tissue was subsequently exposed for 0.5 h at 50 °C to formaldehyde vapour from paraformaldehyde powder equilibrated with 60% relative air humidity and then re-exposed for 1 h at 80 °C. Thus the tissue is first exposed under relatively mild conditions according to the rule of 110 (the temperature in degrees centigrade plus the relative humidity of air in per cent with which the paraformaldehyde powder has been equilibrated must be 110; in the present case 50 + 60), which has been found to develop fluorescence without a risk of diffusion[8]. Subsequent exposure at a

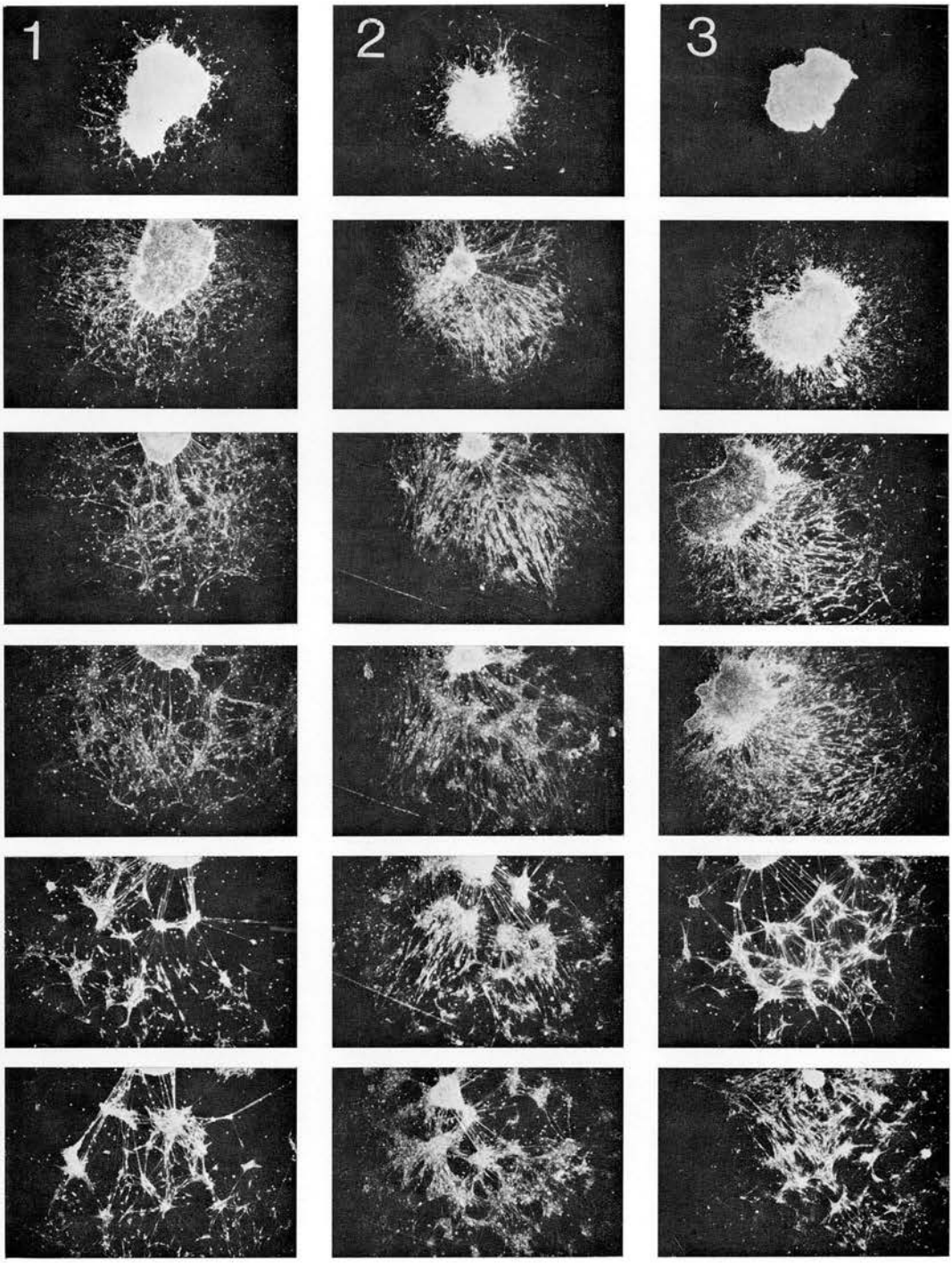


Fig. 1. A vertical series of 6 photomicrographs illustrating the growth of a typical control explant after culture for 2, 4, 6, 8, 10 and 13 days. Dark ground microscopy. × 15.

Fig. 2. A series taken in the same way as that in Fig. 1 of a simultaneous culture containing 9 mg/l of guanethidine sulphate for 6 days (top 3 photographs) and control medium for the following 7 days (bottom 3 photographs). Note that the growth rate and patterns are similar to those in Fig. 1. × 15.

Fig. 3. The upper 4 photographs show the growth of an explant cultured with 36 mg/l of guanethidine sulphate for 1, 3, 5 and 7 days. Note that the growth is normal even at this high concentration. The 2 lower photographs show outgrowth from explants after culture with 1 or 3 mg/l of guanethidine sulphate for 6 days and control medium for the following 7 days, to be compared with the last photographs in Figs. 1 and 2. × 15.

higher temperature then brings out maximum fluorescence in the specimen in which the catecholamines have been immobilized by the previous exposure.

The fluorescence was examined and photographed with a Leitz Ortholux microscope fitted with an HBO 200 high-pressure mercury burner, a filter set consisting of one 3 mm BG 38, two 3 mm BG 12 and one K 530 filter, as well as the Orthomat automatic microscope camera. Transmitted light was used.

RESULTS

General growth patterns

Controls. Fig. 1 shows a series of dark-ground photomicrographs of a control explant of a sympathetic ganglion taken after 2, 4, 6, 8, 10 and 13 days of culture. By 2 days, all explants were surrounded by fibres and cells to approximately the same extent in all directions. This type of growth proceeded in the same manner for several days, during which time the outgrowth still appeared equally dense from the explant border to the periphery. Thereafter, the outgrowth became divided into an inner zone of radial fibres near the explant and an outer zone rich in cells. A few days later, lateral zoning of the outgrowth was frequently seen, resulting in the formation of cellular islands connected with fibres. Further division of such islands occurred during subsequent outgrowth.

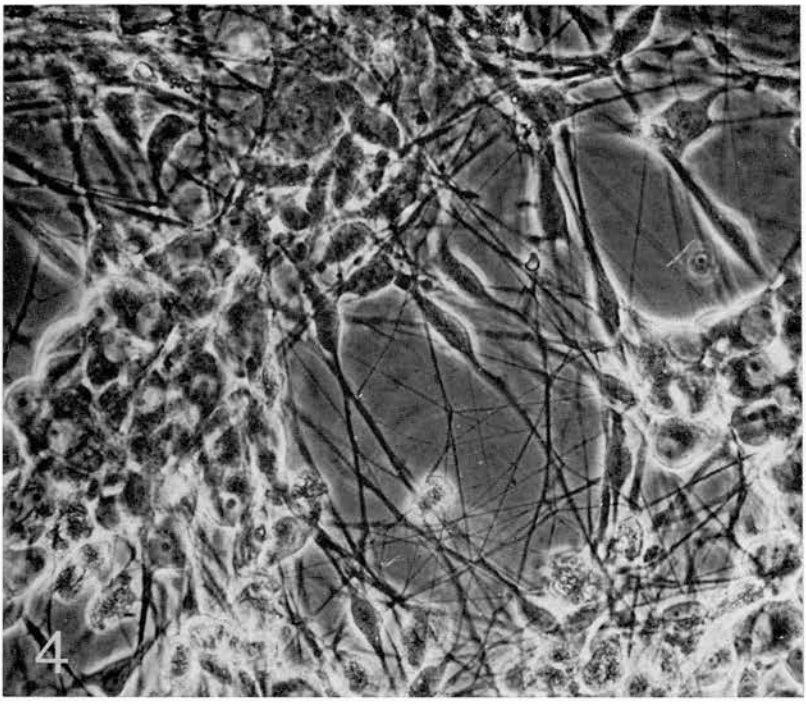
The explants sometimes appeared unchanged during the culture period. However, other explants flattened out to form a thin layer of cells. Such flattened explants either remained spread out or became thicker again so that their internal structure was hard to observe. All types of explant could be seen in one and the same chamber, without any clear reason for differences between the individual explants. Although variations in the explant thickness made accurate volume estimations impossible, it appeared that there was little change in the explant volume during the period observed.

Guanethidine. Successive dark-ground photomicrographs of cultures containing guanethidine (1–36 mg/l) are shown in Figs. 2 and 3. The general features described above for controls also applied to the guanethidine-containing cultures. They appeared normal irrespective of the origin of the explant (superior cervical or thoracic chain ganglia) or the concentration of guanethidine. The rate of growth was likewise well within control limits. The explants, which showed varying degrees of flattening or rounding, appeared to remain essentially unchanged in size.

Cellular structures

Controls. Cell types in normal cultures of sympathetic ganglia have been described in detail elsewhere[4,5]. In our paper, the presentation is limited to features which are essential for judging the effect of guanethidine.

In the flattened explants, the nerve cell bodies were clearly distinguishable by their vesicular nuclei with dense nucleoli and large granular perikarya (Fig. 5).



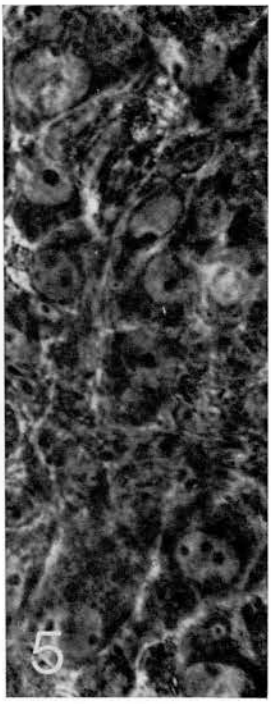


Fig. 4. Phase contrast photomicrograph of groups of nerve cell bodies, nerve fibres and supporting cells near explant cultured for 7 days in the control medium. × 400.
Fig. 5. Nerve cell bodies inside an explant cultured for 7 days in the control medium. × 640.

Occasionally, groups of nerve cells were seen to extend a short distance out of the main explant from which emerged fibres of varying diameter (Fig. 4). These fibres branched and extended to the periphery of the outgrowth (Fig. 8). Spindle-shaped Schwann cells with long cytoplasmic processes were closely associated with the fibre network. These structures together with fibroblasts, glial cells and macrophages formed the cellular islands in the outgrowth described above (Fig. 1). Nerve cell bodies were never seen in these islands.

Guanethidine. No significant differences from normal were observed in the cellular appearances of any of the guanethidine-containing cultures. Outgrowth of supporting cells, Schwann cells and nerve fibres in cultures with 36 mg/l of guanethidine (Fig. 9) showed patterns exactly similar to those in the control cultures (Fig. 8). Likewise, the nerve cell bodies in the explants (Figs. 6 and 7) appeared completely normal after 7 days of culture even with 36 mg/l of guanethidine. In all the 5 experiments, in which the development of the control and guanethidine cultures were daily compared, guanethidine did not seem to have any effect on the growth of the ganglion explants.

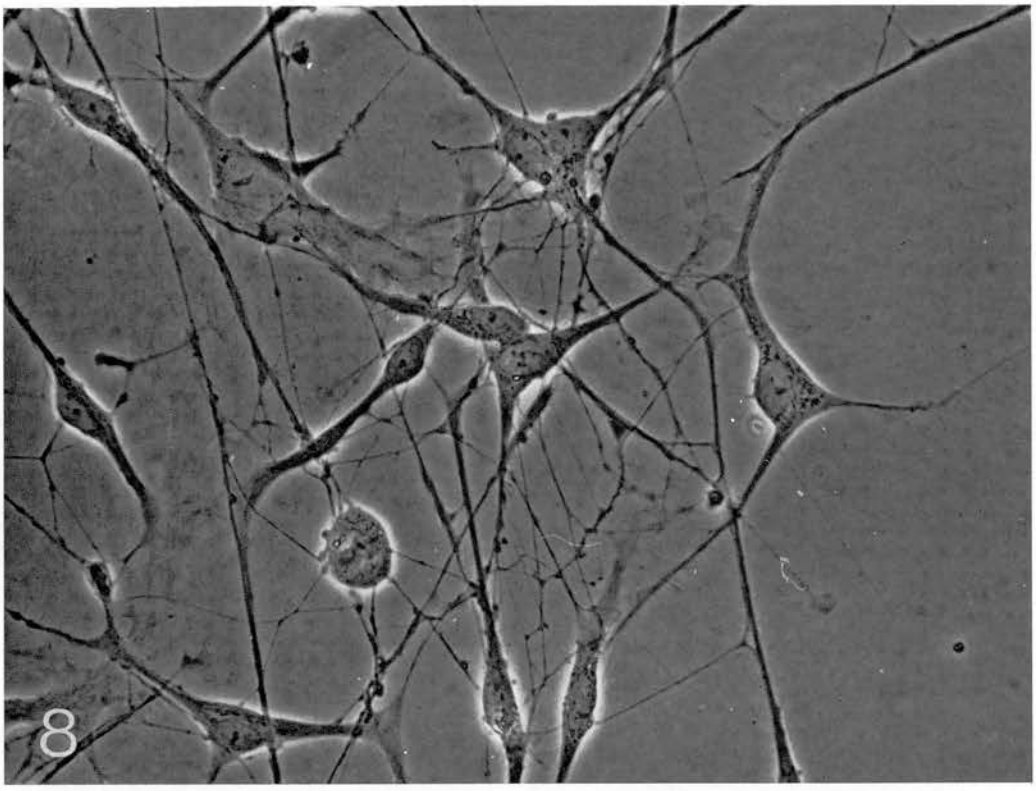
Fig. 6. Peripheral region of an explant cultured with 36 mg/l of guanethidine sulphate for 7 days showing normal nerve cell bodies, nerve fibres and supporting cells. × 400.
Fig. 7. Nerve cell bodies inside an explant cultured for 5 days with 36 mg/l of guanethidine sulphate. × 640.

Formaldehyde-induced fluorescence

Controls. In experiment 1 the control cultures of the superior cervical ganglion showed great variability with regard to SIF cells. Some explants showed several SIF cells, while many showed few or none. This is understandable, because the SIF cells are irregularly distributed in the superior cervical ganglion and the small pieces dissected from it for culture therefore contained several, few or no SIF cells. Experiments 2–5 were indeed therefore carried out with thoracic chain ganglia, the cultures of which showed SIF cells as a more regular feature, although the number of SIF cells varied from one explant to another and the small explants were sometimes without any SIF cells.

Fig. 10 shows the distribution of catecholamine fluorescence in a typical control explant. Against a weakly fluorescent background, small- and medium-sized clusters of SIF cells were seen exhibiting a moderate green fluorescence. Large areas of all control explants were devoid of SIF cells.

Ordinary nerve cells and their processes showed only a very weak fluorescence, the specific nature of which was uncertain. However, SIF cells and even their fine fibres exhibited a strong green catecholamine fluorescence, indicating the validity of the histochemical method.



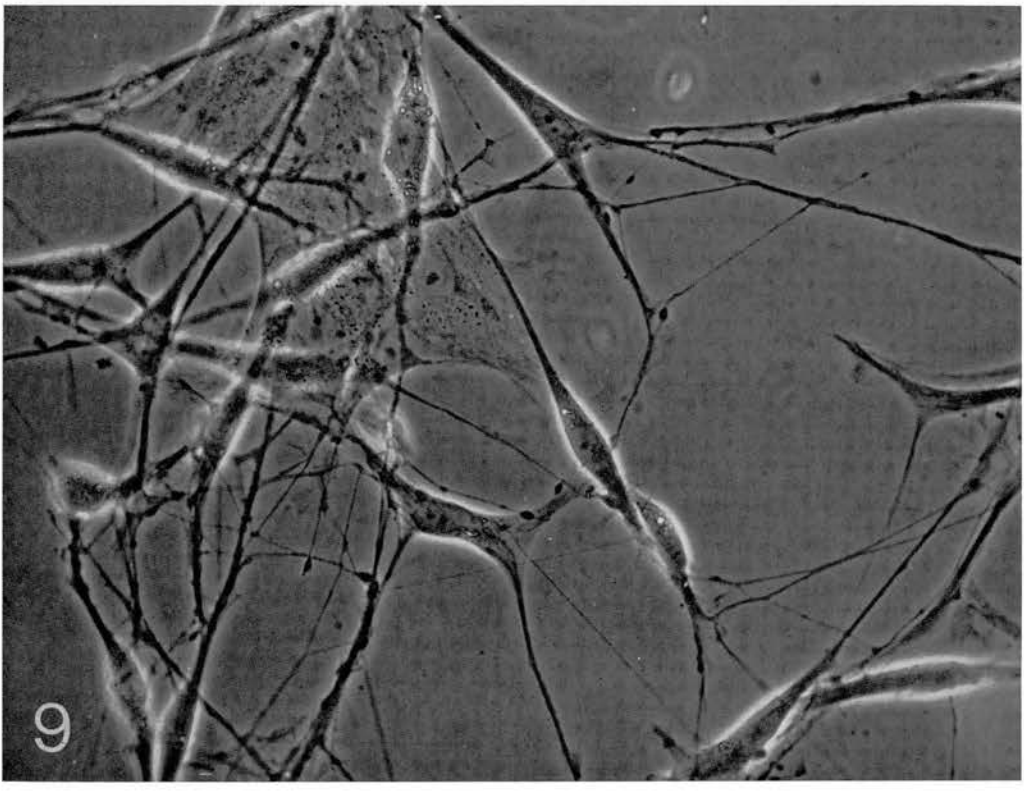


Fig. 8. Fine nerve fibres and glial cells in the periphery of the outgrowth of an explant cultured for 5 days in the control medium. Phase contrast. × 640.
Fig. 9. Similar field from a 7-day-old culture containing 36 mg/l of guanethidine sulphate. Nerve fibres and supporting cells appear normal. × 640.

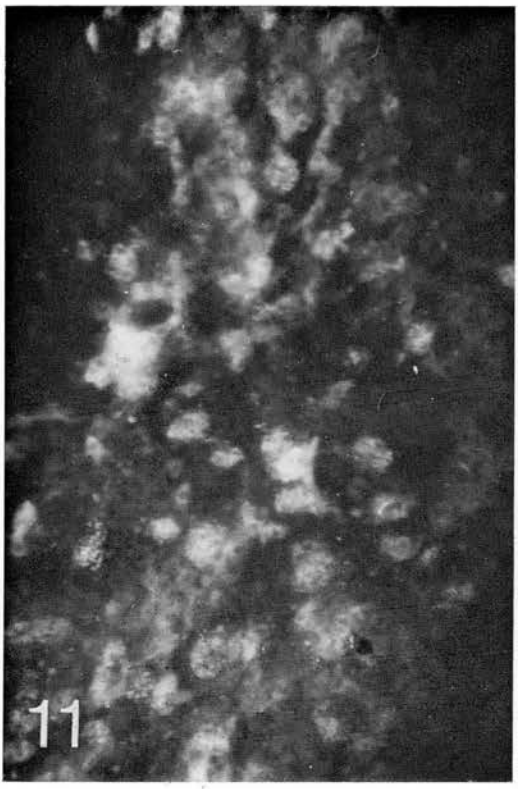
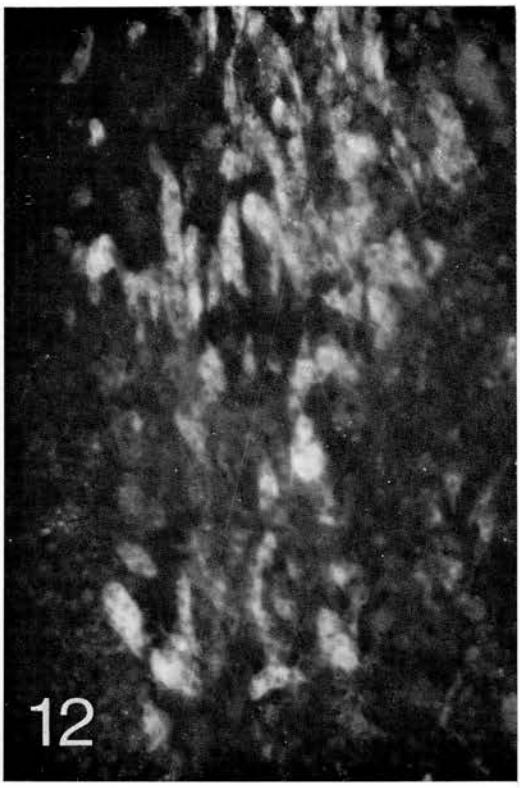


Fig. 10. Formaldehyde-induced fluorescence in an explant cultured for 8 days in the control medium. Moderately fluorescent SIF cells and clusters of them are seen against a weakly fluorescent explant. × 160.

Fig. 11. Fluorescence photomicrograph of an explant cultured with 1 mg/l of guanethidine sulphate for 8 days. Note the great increase in the number of SIF cells, which are distributed more evenly throughout the explant. × 160.

Guanethidine. The number of SIF cells was distinctly increased after culture with 1 mg/l of guanethidine sulphate (Fig. 11) as compared with the control (Fig. 10). Small clusters of SIF cells often covered the whole explant. This increase was quite clearly observed in all experiments in which this dose was used: Experiment 1, 6 days culture of the superior cervical ganglion; experiment 2, 8 days culture of the thoracic chain ganglia; experiment 3, 6 days culture of thoracic chain ganglia; experiment 4, 6 days culture of thoracic ganglia with guanethidine and thereafter further 7 days in the control medium. The intensity of the fluorescence was elevated as well but the colour of the fluorescence remained green.

With higher concentrations of guanethidine in the culture medium, great variation was observed in the number and fluorescence intensity of the SIF cells in each explant. In experiments 1 and 2, for example, cultures with 3 mg/l showed an increased number of SIF cell clusters (Fig. 12) and those with 9 mg/l exhibited but few weakly fluorescent SIF cells, while few SIF cells were seen in experiments 3 and 4



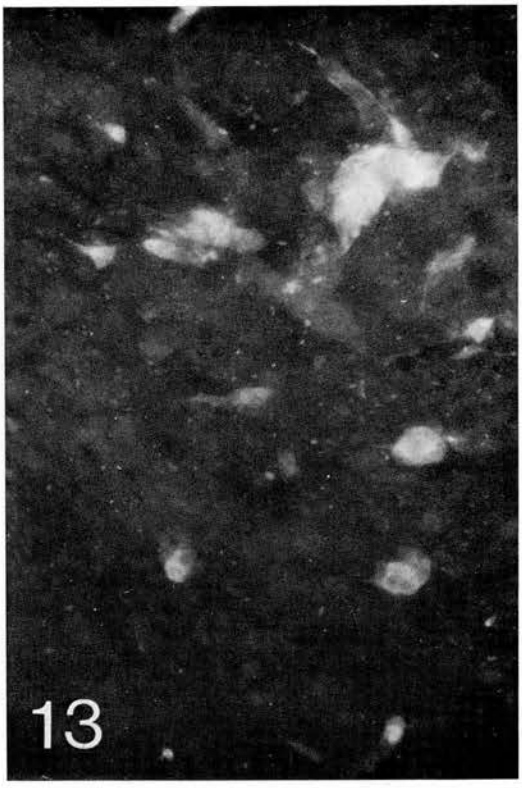


Fig. 12. Similar photograph of an explant cultured with 3 mg/l of guanethidine sulphate for 8 days. A distinct increase in the number of SIF cells is seen. × 160.
Fig. 13. Explant cultured with 36 mg/l of guanethidine sulphate for 7 days. Scattered SIF cell clusters are present, although their number is much smaller than those in Figs. 11 and 12. × 160.

in cultures with either 3 or 9 mg/l. Surprisingly, many explants in experiment 5 with 36 mg/l of guanethidine showed SIF cell clusters with a more intense fluorescence (Fig. 13) than that in the controls.

DISCUSSION

In the present study, guanethidine sulphate caused, even in the lowest concentration (1 mg/l), an increase in the number of SIF cells in the ganglion explants. This is in keeping with the previous observation[7] that injections of guanethidine into newborn rats cause an increase in the number of SIF cells in the sympathetic ganglia and suggests that guanethidine has a direct action on cultured SIF cells or their precursors. It is of interest in this connexion that hydrocortisone has also been shown to cause an increase in the number of SIF cells both *in vivo*[6] and *in vitro*[11].

In concentrations higher than 1 mg/l, guanethidine sulphate caused variable effects on the number of SIF cells and their fluorescence intensity. It seems likely that guanethidine has two kinds of direct effect on the SIF cells: an increase in the number

of these cells and a depletion of catecholamines from them. Therefore, since the SIF cells were identified in the present study solely on the basis of their fluorescence, a possible increase in the number of SIF cells due to higher doses of guanethidine may have remained unnoticed because of simultaneous depletion of catecholamines from them.

In our study, nerve cells in control cultures did not seem to contain appreciable amounts of histochemically demonstrable catecholamines. This seems to indicate a loss of catecholamines from the nerve cells during the culture period, since sympathetic neurones of newborn rats are known to exhibit catecholamine fluorescence[10]. However, fluorescence has been demonstrated in cultures of ganglia from 5- to 20-day-old rats[5], presumably because a more advanced stage of neuronal differentiation results in an improved uptake and storage mechanism.

While guanethidine affected the SIF cells in a similar way *in vivo* and *in vitro* its effects on the nerve cells were entirely different under these two conditions. Eight daily injections of 20 mg/kg of guanethidine sulphate cause destruction of sympathetic ganglia in newborn rats[7], 14 similar injections cause serious degenerative changes in the sympathetic ganglia of adult rats[7,19], while 25 mg/kg of the same drug given daily for 6 weeks results in destruction of sympathetic ganglia in adult rats[3]. In contrast, in the present study, the nerve cells in the cultures of sympathetic ganglia of newborn rats not only survived for a week in the presence of as much as 36 mg/l of guanethidine sulphate but also appeared normal at the light microscope level and formed a nerve fibre net at the same rate as the neurones in the control cultures.

Further considerations seem to suggest that the maximum concentration of guanethidine attained in the ganglia of living animals is lower than that used in some of our cultures. Juul and Sand[20] showed that daily administration of guanethidine into adult rats results in a gradual increase of its concentration in the superior cervical ganglion. Maximum concentration of 56 ng/ganglion (dry weight 0.6–0.7 mg) was reached after 14 days' daily administration of 60 mg/kg of guanethidine sulphate. Further increase in the dose or the length of the treatment failed to increase the concentration of the drug in the ganglion. Assuming a water content of 80% in the ganglion, the maximum concentration was about 56 ng/3 mg of fresh tissue or 19 mg/kg. Since a concentration of 36 mg/l of the same drug was maintained in the culture medium in our experiments, it appears that the cultured sympathetic nerve cells were surrounded by a higher concentration than that reached *in vivo* inside the ganglion under any dosage condition.

As a result of neuronal uptake of guanethidine, the concentration within the nerve cells may in fact be higher than that outside them[27]. If it is assumed that cultured nerve cells are capable of concentrating guanethidine in the same way as nerve cells *in vivo*, then the guanethidine concentration inside the former should be higher than that inside the latter. However, a failure in the uptake mechanism *in vitro* could prevent accumulation of guanethidine in sufficient concentration to cause destruction. If this were so, the same failure should also apply to the *in vitro* uptake of 6-hydroxydopamine, whose neurotoxic effect *in vivo*[2] is due to its accumulation inside the nerve

cells[26]. However, exposure of mouse neuroblastoma cultures to 6-hydroxydopamine for only 1 h causes marked degenerative changes and repeated 1 h exposures during 3 consecutive days results in the complete disappearance of the nerve cells[1]. Thus, it seems unlikely that guanethidine has a direct toxic effect like 6-hydroxydopamine.

If guanethidine does not exert a direct action on the sympathetic nerve cells, and further studies are required to exclude, for example, the possibility of a direct but very slow toxic effect, what then could be the mechanism by which it does cause destruction of the sympathetic nerve cells in living animals? In this connexion, it is of interest to recall that guanethidine causes a marked round cell infiltration of the sympathetic ganglia *in vivo*[7,19]. This phenomenon is possibly of immunological origin[19] in that it resembles accumulation of small cells in the central nervous system of animals with an experimental allergic encephalomyelitis[21]. Against this background, it is possible that guanethidine causes a selective liberation of antigenic substances from sympathetic nerve cells, an autoimmune reaction and a selective destruction of the same nerve cells. Antiserum against the nerve growth factor, indeed, selectively destroys sympathetic ganglion cells of newborn rats in a way[22] that closely resembles the destruction caused by guanethidine[7]. In cultures of nerve cells an immune reaction is less likely to occur in the absence of sufficient numbers of antibody-forming cells.

The possibilities also exist that guanethidine, outside the nervous system, is converted into a toxic metabolite or causes the formation of a toxic substance, either of which may subsequently cause destruction of the sympathetic nerve cells. Such a view can be supported by the fact that guanethidine is taken up not only by sympathetic nerve cells but by cells of other tissue as well[27].

SUMMARY

Pieces of sympathetic ganglia of newborn rats were cultivated in modified Rose chambers in Medium 199 supplemented with serum, insulin, penicillin and glucose. Guanethidine sulphate was added to make the following concentrations in the medium: 0 (controls), 1, 3, 9 or 36 mg/l. The cultures were daily examined using dark ground and phase contrast microscopy. After 6–8 days of culture with guanethidine, the catecholamines were demonstrated with the formaldehyde-induced fluorescent method.

There was a clear increase in the number of the small intensely fluorescent cells in cultures with 1 mg/l of guanethidine sulphate, suggesting that it has a direct effect on these cells. Cultures with higher concentrations showed variability in the number of these cells, probably due to depletion of their catecholamine content.

Light microscopic appearances of guanethidine-containing cultures did not differ from control cultures in nerve cell structure, nerve fibre growth rate or cellular composition of the outgrowth. It is therefore concluded that guanethidine has no apparent cytotoxic effect on sympathetic nerve cells *in vitro*.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Sigrid Jusélius Foundation to

O.E. and grants from the Australian Research Grants Committee and the National Heart Foundation of Australia to G.B.

We are very grateful to Miss Janet McConnell for skillful technical assistance in the tissue culture laboratory and to Mr. Brian Pump for excellent printing of the photomicrographs.

REFERENCES

1 ANGELETTI, P. U., AND LEVI-MONTALCINI, R., Cytolytic effect of 6-hydroxydopamine on neuroblastoma cells, *Cancer Res.*, 30 (1970) 2863–2869.
2 ANGELETTI, P. U., AND LEVI-MONTALCINI, R., Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine, *Proc. nat. Acad. Sci. (Wash.)*, 65 (1970) 114–121.
3 BURNSTOCK, G., EVANS, B., GANNON, B. G., HEATH, J. W., AND JAMES, V., A new method of destroying adrenergic nerves in adult animals using guanethidine, *Brit. J. Pharmacol.*, 43 (1971) 295–301.
4 CHAMLEY, J. H., MARK, G. E., AND BURNSTOCK, G., Sympathetic ganglia in culture. II. Accessory cells, *Z. Zellforsch.*, (1972) submitted for publication.
5 CHAMLEY, J. H., MARK, G. E., CAMPBELL, G. R., AND BURNSTOCK, G., Sympathetic ganglia in culture. I. Neurons, *Z. Zellforsch.*, (1972) submitted for publication.
6 ERÄNKÖ, L., AND ERÄNKÖ, O., Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat, *Acta physiol. scand.*, 84 (1972) 125–133.
7 ERÄNKÖ, L., AND ERÄNKÖ, O., Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats, *Acta pharmacol. (Kbh.)*, 30 (1971) 403–416.
8 ERÄNKÖ, O., The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence, *J. roy. micr. Soc.*, 87 (1967) 259–276.
9 ERÄNKÖ, O., AND ERÄNKÖ, L., Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats, *Histochem. J.*, 3 (1971) 451–456.
10 ERÄNKÖ, O., AND ERÄNKÖ, L., Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. In O. ERÄNKÖ (Ed.), *Histochemistry of Nervous Transmission, Progress in Brain Research, Vol. 34*, Elsevier, Amsterdam, 1971, pp. 34–51.
11 ERÄNKÖ, O., ERÄNKÖ, L., HILL, C., AND BURNSTOCK, G., Hydrocortisone-induced increase in the number of small intensely fluorescent cells and their histochemically demonstrable catecholamine content in cultures of sympathetic ganglia of the rat, *Histochem. J.*, 4 (1972) 49–58.
12 ERÄNKÖ, O., AND HÄRKÖNEN, M., Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat, *Acta physiol. scand.*, 58 (1963) 285–286.
13 ERÄNKÖ, O., AND HÄRKÖNEN, M., Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them, *Acta physiol. scand.*, 63 (1965) 511–512.
14 GANNON, B. J., IWAYAMA, T., AND BURNSTOCK, G., Prolonged effects of chronic guanethidine treatment on the sympathetic innervation of the genitalia of male rats, *Med. J. Aust.*, 207 (1971) 207–209.
15 HANKS, J. H., AND WALLACE, R. E., Relation of oxygen and temperature in the preservation of tissues by refrigeration, *Proc. Soc. exp. Biol. (N.Y.)*, 71 (1949) 196–200.
16 HEATH, J. W., EVANS, B. K., GANNON, B. J., BURNSTOCK, G., AND JAMES, V. B., Degeneration of adrenergic neurons following guanethidine treatment: an ultrastructural study, *Virchows Arch. exp. Path.* (1972) in press.
17 JENSEN-HOLM, J., Cholinesterase and protein changes in sympathetic ganglia after guanethidine, *Acta pharmacol. (Kbh.)*, 25, Suppl. 4 (1967) 74.
18 JENSEN-HOLM, J., AND JUUL, P., The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and protein, *Acta pharmacol. (Kbh.)*, 28 (1970) 270–282.
19 JENSEN-HOLM, J., AND JUUL, P., The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and catecholamines (histochemistry), and histology, *Acta pharmacol. (Kbh.)*, 28 (1970) 283–298.

20 JUUL, P., AND SAND, O., Guanethidine determination in rat sympathetic ganglia following prolonged administration, *Acta pharmacol. (Kbh.)*, 29, Suppl. 4 (1971) 25.
21 LAMPERT, P. W., Demyelination and remyelination in experimental allergic encephalomyelitis. Further electron microscopic observation, *J. Neuropath. exp. Neurol.*, 24 (1965) 371–385.
22 LEVI-MONTALCINI, R., AND ANGELETTI, P. U., Immunosympathectomy, *Pharmacol. Rev.*, 18 (1966) 619–628.
23 MATTHEWS, M. R., AND RAISMAN, G., The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion, *J. Anat. (Lond.)*, 105 (1969) 255–282.
24 ROSE, G. C., A separable and multipurpose tissue culture chamber, *Tex. Rep. Biol. Med.*, 12 (1954) 1074–1083.
25 SALK, E. S., YOUNGNER, J. S., AND WARD, E. N., Use of color change of phenol red as the indicator in titrating poliomyelitis virus or its antibody in a tissue culture system. Appendix. Method of preparing medium 199, *Amer. J. Hyg.*, 60 (1954) 214–230.
26 SANER, A., AND THOENEN, H., Model experiments on the molecular mechanism of action of 6-hydroxydopamine, *Molec. Pharmacol.*, 7 (1971) 147–154.
27 THOENEN, H., *Bildung und funktionelle Bedeutung adrenerger Ersatztransmitter*, Springer, Berlin, 1969, pp. 1–85.

Z. Zellforsch. 134, 297—310 (1972)

Effect of Hydrocortisone on the Ultrastructure of the Small, Intensely Fluorescent, Granule-containing Cells in Cultures of Sympathetic Ganglia of Newborn Rats*

Olavi Eränkö**, John Heath***, and Liisa Eränkö△

Department of Zoology, University of Melbourne, Parkville, Victoria, Australia
(Head: Prof. G. Burnstock, D. Sc.)

Received August 14, 1972

Summary. Sympathetic chain ganglia of newborn rats were cultured in Rose chambers with or without hydrocortisone. After one week, the cultures were examined by light microscopy for formaldehyde-induced catecholamine fluorescence and by electron microscopy after fixation in 5% glutaraldehyde solution and thereafter in 1% osmium tetroxide. Hydrocortisone (10 mg/l) caused a great increase in the number of the small, intensely fluorescent (SIF) cells in the ganglion explants, and the fluorescence intensity of these cells was also increased. The SIF cells corresponded to small, granule-containing (SGC) cells in the electronmicros copic preparations, and in addition to an increase in their number there was also an increase in the size and number of granular vesicles in the presence of hydrocortisone. In control cultures the granular vesicles were round (about 100 nm in diameter) or elongated (40-150 nm in cross section and 150–250 nm in length); both types of vesicles contained electron dense cores. In hydrocortisone-containing cultures round granular vesicles up to 200 nm in diameter were also observed; the cores of these vesicles were of variable electron density. It is concluded that in tissue culture, hydrocortisone causes an increased formation of catecholamine-containing granular vesicles in SIF-SGC cells and their precursors and an increase in the number of these cells.

Key words: Sympathetic Ganglia — Tissue culture — Catecholamines — SIF cells — Granular vesicles — Hydrocortisone — Ultrastructure.

Introduction

Increasing attention has recently been paid to a special type of sympathetic cell, which was first identified in the superior cervical ganglion of the rat on the bases of its small size and extremely bright formaldehyde- induced amine fluorescence (Eränkö and Härkönen, 1963). These cells, now often called "small, intensely fluorescent" (SIF) cells (Eränkö and Härkönen, 1965a), have subsequently been observed by fluorescence microscopy in various sympathetic ganglia of several

* This work was supported by grants from the National Heart Foundation, the Australian Research Grants Committee and the Sigrid Juselius Foundation.

** University of Melbourne Senior Research Fellow, September, 1971 — August, 1972; present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland, 00170.

*** Holder of a grant from the National Health and Medical Research Council of Australia.

△ Sunshine Foundation and Rowden White Research Fellow in the University of Melbourne, September, 1971 — August, 1972; present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland, 00170.

species (Norberg and Hamberger, 1964; Eränkö and Härkönen, 1965b; Owman and Sjöstrand, 1965; Norberg *et al.*, 1966; Norberg and Sjöqvist, 1966; Csillik *et al.*, 1967; Jacobowitz, 1967; Olson, 1967; Van Orden *et al.*, 1970; Kanerva, 1971). Cells of the same size and shape as the SIF cells have been found to contain, like cells of the adrenal medulla, numerous granular vesicles (Eränkö and Härkönen, 1965b; Grillo, 1966; Williams, 1967; Elfvin, 1968; Siegrist *et al.*, 1968; Hökfelt, 1969; Matthews and Raisman, 1969; Taxi *et al.*, 1969; Williams and Palay, 1969; Van Orden *et al.*, 1970; Watanabe, 1970; 1971; Eränkö, 1972; Kanerva, 1972; Kanerva and Teräväinen, 1971). Accordingly, these cells have been called "small granule-containing" (SGC) cells (Matthews and Raisman, 1969). It is now apparent (Jacobowitz, 1970; Van Orden *et al.*, 1970; Eränkö and Eränkö, 1971), that the SIF cells are probably identical with the SGC cells, the intense foramaldehydeinduced fluorescence being due to the presence of high concentrations of catecholamines in the cytoplasmic granules of these cells.

In the sympathetic ganglia of newborn rats hydrocortisone administration causes a great increase in the number of SIF cells (Eränkö and Eränkö, 1972) and SGC cells (Eränkö *et al.*, 1972). In cultures of sympathetic ganglia, in which the SIF cells readily survive (Chamley *et al.*, 1972b), addition of hydrocortisone into the culture medium causes not only an increase in the number of the SIF cells but also a marked increase in the intensity of their formaldehyde-induced fluorescence (Eränkö *et al.*, 1972a). Typical SGC cells have been described in cultures of sympathetic ganglia (Lever and Presley, 1971), but the effect of hydrocortisone on their ultrastructure has not been studied *in vitro*. Such a study was therefore carried out on newborn rat sympathetic ganglia.

Materials and Methods

Newborn albino rats of the Sprague-Dawley strain were used. Since it is known that the effect of cortisone and hydrocortisone on extra-adrenal chromaffin tissue and sympathetic ganglia depends on an early developmental stage (Lempinen, 1964; Eränkö and Eränkö, 1972), only animals born in the previous night or the same day were used. After killing the animals by a blow on the head, the superior cervical ganglia and/or the thoracic and abdominal paravertebral sympathetic ganglia were dissected under sterile conditions, carefully avoiding excessive handling of the ganglia. These were then treated with 0.125% trypsin for 20 min.

Details of the method of tissue culture employed have been described by Chamley *et al.* (1972a). Here it is sufficient to say that the ganglia were collected in a salt solution containing penicillin and streptomycin, placed on collagen-coated coverslips, covered with strips of dialyzing cellophane and cultured in modified Rose (1954) chambers in a culture medium 199 (Salk *et al.*, 1954) supplemented with 20% (v/v) of foetal calf serum, 0.05 units/ml of insulin, 100 units/ml of penicillin G, 5 mg/ml of glucose and 1 unit/ml of nerve growth factor (Burroughs and Wellcome, England). Hydrocortisone sodium succinate (Efcortelan soluble, Glaxo-Allenburys, Australia, Pty. Ltd.) was added to the experimental culture medium to make a final concentration of 0.01 mg/ml of hydrocortisone. The chambers were kept at 37°C in an incubator supplied with a continuous flow of 5% of carbon dioxide in air bubbled through water.

The culture medium was changed daily, and the cultures were examined by phase contrast microscopy. After 7 days of culture, the chambers were opened, and some cultures were dried in a vacuum desiccator and exposed to paraformaldehyde vapour for histochemical demonstration of catecholamines by fluorescence microscopy (Eränkö, 1967; for details of handling the tissue cultures, see Eränkö *et al.*, 1972a). For fluorescence microscopy a Leitz Ortholux microscope was used with an HBO 200 mercury lamp and the filter combination of BG 12 and

K 510. Other cultures were prepared for electron microscopy as follows. The cultures on the coverslip were immersed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 mins. They were then washed in the buffer for 10 min and post-fixed in phosphate-buffered 1% osmium tetroxide for 30 min. After a brief rinse in distilled water the cultures were stained in a 2% aqueous solution of uranyl acetate for 1 hour, and dehydrated in a graded acetone series. The cultures were then infiltrated in a mixture of equal volumes of acetone and Araldite and embedded in Araldite. Thin sections cut with a Huxley-Cambridge ultramicrotome were double stained with a saturated aqueous solution of uranyl acetate followed by lead citrate, and examined in a Hitachi 11 B electron microscope.

Seven experiments were carried out, each consisting of 12–16 control cultures and 18–24 cultures with 10 mg/l of hydrocortisone added to the culture medium.

Observations

Catecholamine Fluorescence

Control Cultures. The control cultures regularly contained SIF cells, but the number of these cells varied from one explant to another. Some explants contained only one small cluster of SIF cells, while others contained one or several large clusters. The fluorescence colour of all the SIF cells in the control cultures appeared green through the yellow filter K 510 employed. The fluorescence intensity of the SIF cells ranged from weak to moderate. No fluorescence was seen in other cells in the ganglia.

Hydrocortisone-containing Cultures. A great increase in the number and fluorescence intensity of the SIF cells was observedin all hydrocortisone-containing cultures. Large clusters of SIF cells often covered a major part of the explant area and numerous smaller clusters were observed in the outgrowth as well. The fluorescence intensity of individual SIF cells within a large cluster often varied considerably. The majority fluoresced with an intense green or yellow colour, but a few of the cells showed a moderate green fluorescence and some exhibited an orange or reddish fluorescence.

Fine Structure

Control Cultures. The SGC cells were, in agreement with the fluorescence microscopic observations, usually found in clusters of several cells. Two typical SGC cells are illustrated in Fig. 1. The nuclei show peripheral aggregations of chromatin, a feature by which the SGC cells can be distinguished from the nerve cells, whose nuclei showed less chromatin aggregation. The cytoplasm of the SGC cells contained numerous mitochondria, cisternae of rough endoplasmic reticulum and abundant ribosomes, often in rosettes. Numerous microtubules were present, especially in the cell processes. The most outstanding feature of these cells was the presence in their cytoplasm of granular vesicles. In most SGC cells, these were restricted largely to the periphery of the cytoplasm (Fig. 1), and were more numerous in some cells than others (Fig. 2, cf. 1). While a thin layer of satellite cell cytoplasm sometimes separated SGC cells, the outer cell membranes of these cells were usually in close contact with each other. This often resulted in appearance such as that in Fig. 2, which suggest the presence of a single cell whose perikaryon is full of granular vesicles, although two cells are present. Cells with granular vesicles

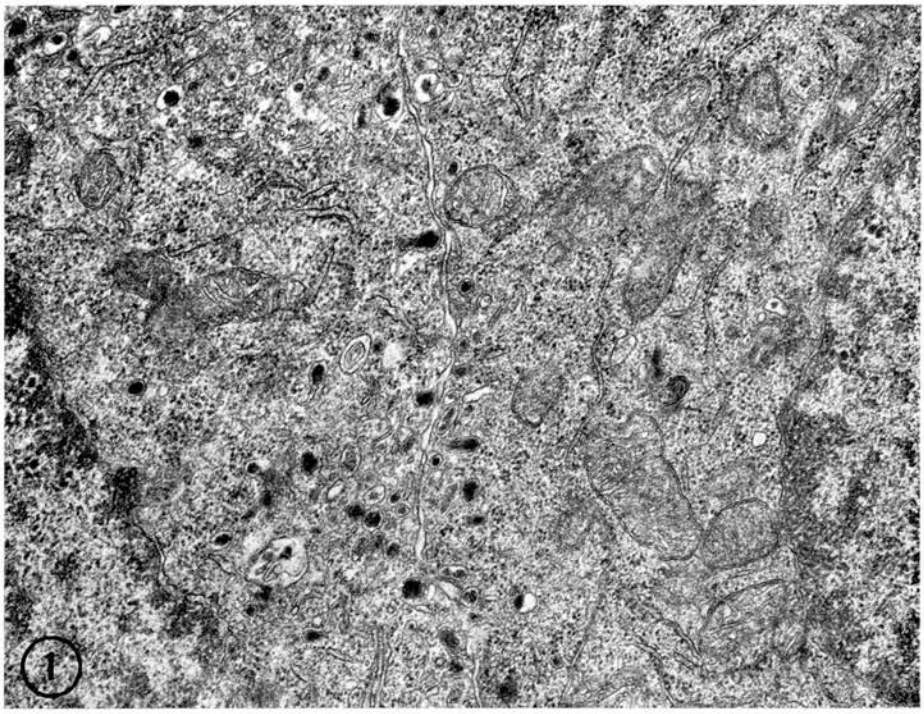


Fig. 1. Two typical SGC cells in control culture. The nuclei show peripheral chromatin aggregation, and the cytoplasm contains an abundance of organelles. The granular vesicles are located mostly in the periphery of the cytoplasm. 7 days culture. ×25000

evenly distributed over the whole cytoplasm were sometimes seen but they were rare. Cell profiles with very few granular vesicles were also observed. Such cells were classified as SGC cells if they showed the size and conformations exhibited by typical SGC cells, and had similar nuclei with peripheral chromatin aggregation. It was not always easy to distinguish between young sympathetic neuroblasts and SGC cells.

The granular vesicles varied both in shape and size. In most cases, the vesicles were almost filled by electron dense material, although an electron-lucent halo about 20 nm wide was always seen between the triple-layered membrane of the vesicle and its electron dense content (Fig. 3). There was some variation in the electron density of the central core but most vesicles showed a high density. Round, oval and elongated vesicles were present, and some tubular structures, also containing a granular substance, were observed (Fig. 3). The diameter of the oval vesicles varied between 40 and 150 nm in cross section and 150–250 nm in length, and were much more common than in SGC cells of sympathetic ganglia from one-week-old animals, in which the granular vesicles were usually round (Eränkö, 1972; Eränkö, *et al.*, 1972b). In elongated vesicles the dense core was sometimes

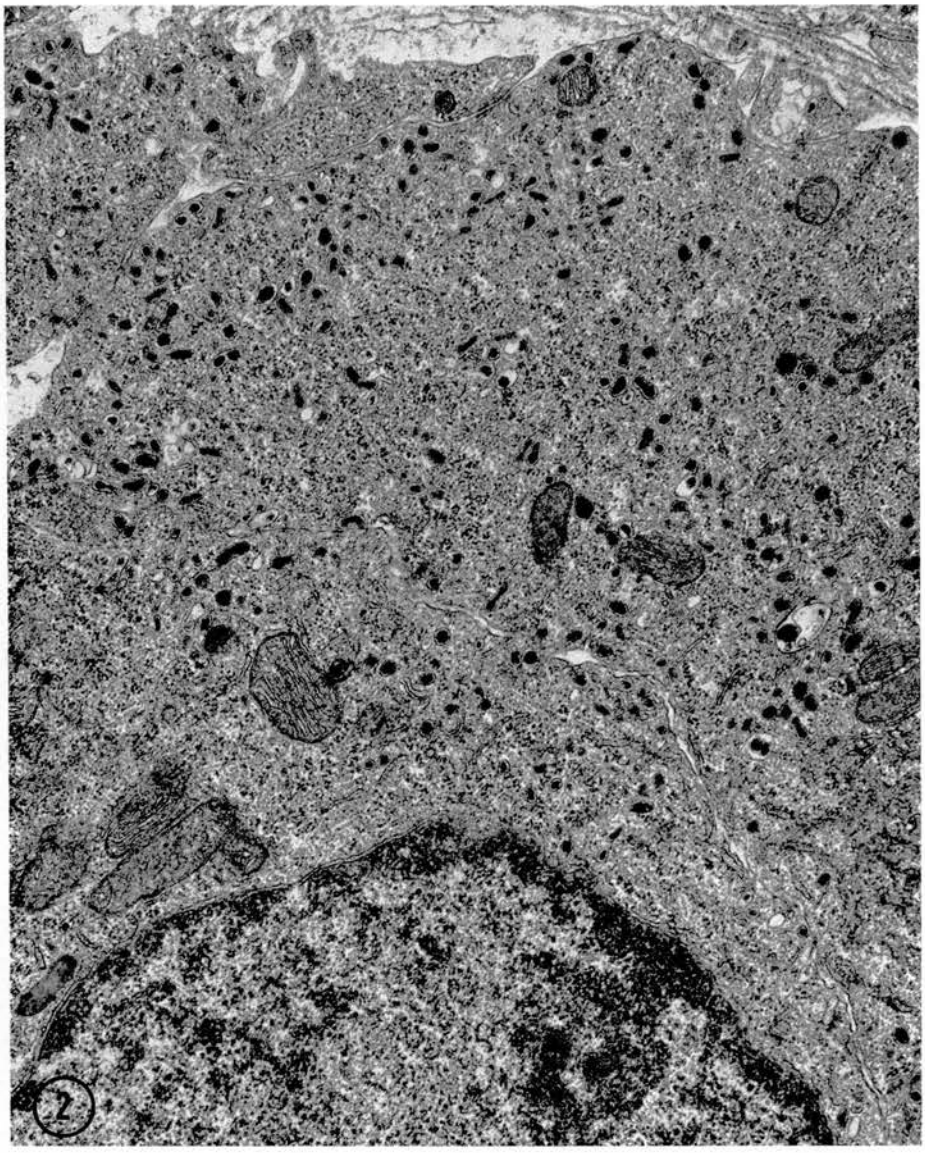


Fig. 2. Close contact between neighbouring SGC cells in a control culture. The upper cell profile contains a larger number of granular vesicles than usually observed in controls. 7 days culture. ×20000

elongated, sometimes hour-glass-shaped, and sometimes consisted of two separate elongated or round granules (Fig. 3).

Hydrocortisone-containing Cultures. A pronounced increase in the number of the SGC cells was observed in hydrocortisone-containing cultures. Some SGC cells

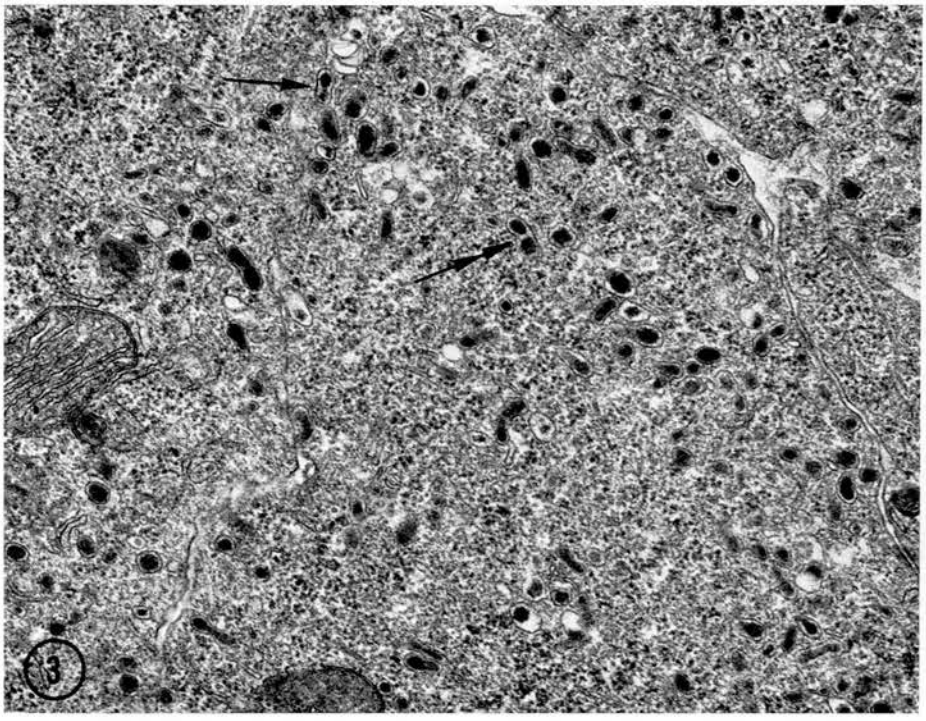


Fig. 3. Higher magnification of an area from Fig. 2. Round, oval and elongated granular vesicles are present. In elongated vesicles the dense core is elongated, hour-glass-shaped (arrow) or consists of two separate granules (double arrow). An electron-lucent halo is visible between the granule and the vesicle membrane. 7 days culture. ×30000

in these cultures resembled those in control cultures, containing about the same number of granular vesicles with similar size (50–150 nm) and peripheral location (Fig. 4). However, most cells in the hydrocortisone-containing cultures showed an increased number of granular vesicles, which were still mostly located in the periphery of the cytoplasm and which were often larger (up to 200 nm in diameter) than those in control cultures (Fig. 5a and b). The cores of some large vesicles were of low electron density and the halo around some granules was very large (Fig. 5b).

Views such as those in Fig. 5 were quite common in the hydrocortisone-containing cultures and suggested that there were two types of cells which essentially differ from each other on the basis of the granule size. However, some fields showed large processes, containing many large (200 nm) granular vesicles, in continuous cytoplasmic contact with a perikaryon containing granular vesicles of about 100 nm in diameter (Fig. 5a, extreme left). Nevertheless, large (200 nm) granular vesicles were sometimes seen in the perikaryon of SGC cells. These vesicles were usually round or oval, and their content was usually very electron-dense. Densely

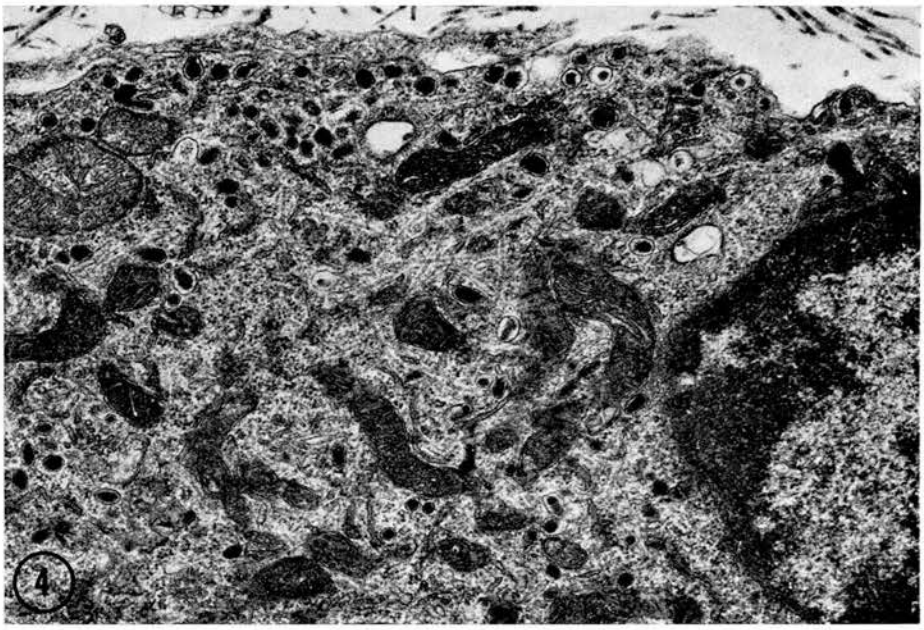


Fig. 4. SGC cell from a hydrocortisone-containing culture. This cell contains a similar number of granular vesicles and has similar ultrastructure to SGC cells in control cultures. 7 days culture with 10 mg/l hydrocortisone sodium succinate included in the culture medium. $\times 20000$

packed round and elongated vesicles about 100 nm in diameter or 80×150 nm in cross section and length were seen in other SGC cell processes (Fig. 6).

In the hydrocortisone-treated cultures the large number and variety of granular vesicles facilitated observation of structures possibly representing successive stages in the cytoplasmic formation of the vesicles. Round, oval and elongated vesicles almost filled with electron-dense granules were observed together with elongated and hour-glass-shaped vesicles containing two separate "granules". Typical granular vesicles were often observed in close association with the Golgi apparatus, and electron dense material was often seen inside apparently typical Golgi vesicles (Fig. 7).

Discussion

The small cells observed in control cultures showed the characteristic features of typical SIF or SGC cells; i.e. formaldehyde-induced cytoplasmic fluorescence and numerous granular vesicles respectively. The presence of numerous mitochondria and ribosomes and well-developed Golgi complexes suggests that these cells are in a state of active metabolism and synthesis. Similar observations were made on SGC cells in cultures of sympathetic ganglia of chicken embryos by Lever and Presley (1971).

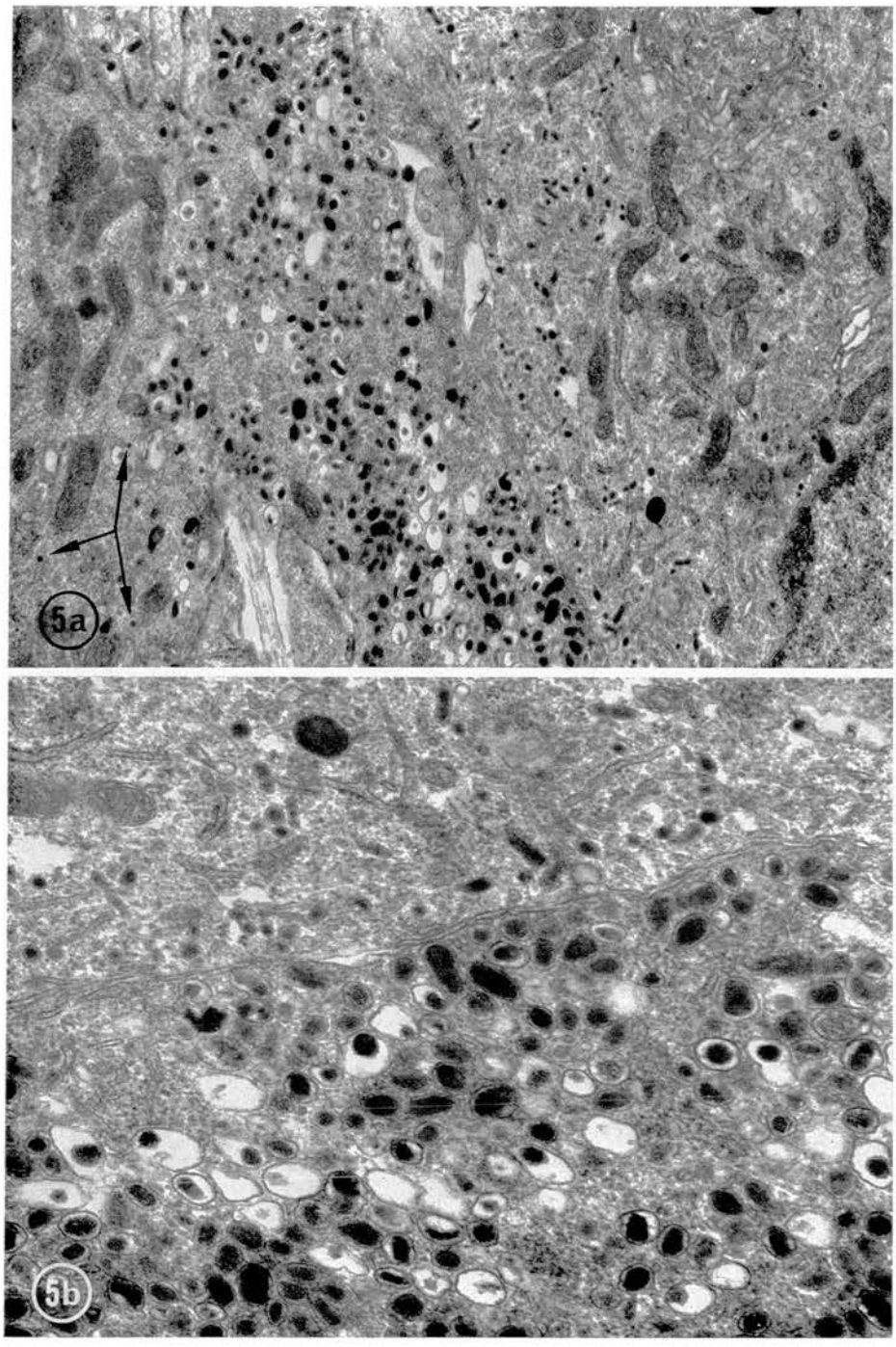


Fig. 5a and b. Areas of two SGC cells from a hydrocortisone-containing culture. a) The periphery of the cell at right contains granular vesicles 50–100 nm in diameter. A process of the cell at left is seen in the centre of this field; the perikaryon of this cell contains some vesicles with a diameter of about 100 nm (arrows) while its process contains a large number of vesicles

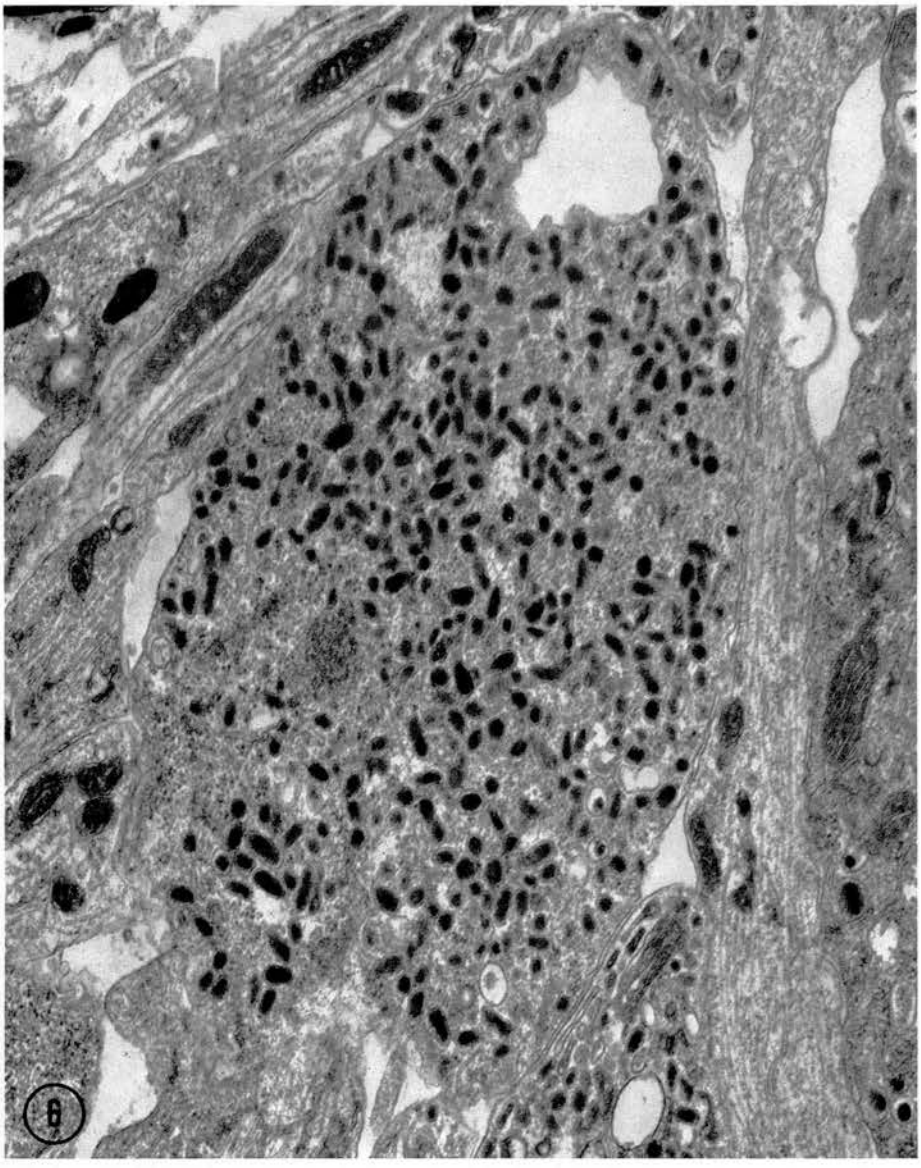


Fig. 6. Process of an SGC cell from a hydrocortisone-containing culture containing a large number of round (diameter 100 nm) and elongated vesicles (width 80 nm × length 150 nm). 7 day culture with 10 mg/l hydrocortisone sodium succinate included in the culture medium. ×30000

with diameter up to 200 nm. ×12000. b) Higher magnification of an area from Fig. 5a. Only minor variation in electron density is apparent in the cores of the 50–100 nm diameter granular vesicles in the upper cell. In the lower cell, great variability is shown in the electron density of the cores of the 200 nm diameter granular vesicles, and in the size of the halos surrounding the cores. ×30000. 7 days culture with 10 mg/l hydrocortisone sodium succinate included in the culture medium

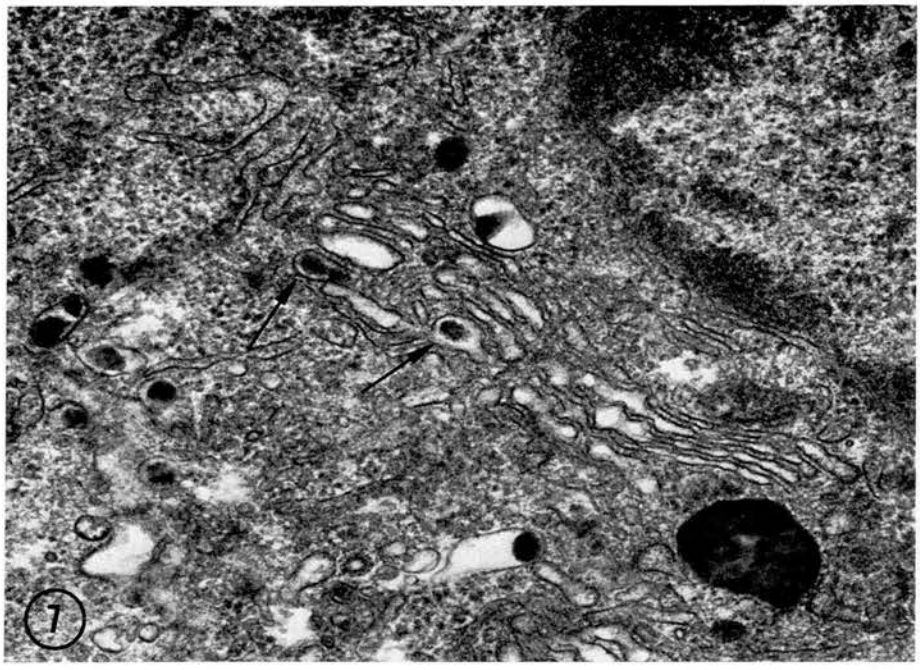


Fig. 7. Perikaryon of an SGC cell from a hydrocortisone-treated culture. Electron-dense granules are present in some vesicles of the Golgi apparatus (arrows). 7 day culture with 10 mg/l hydrocortisone sodium succinate included in the culture medium. ×45000

The round granular vesicles observed in the control cultures were 50–150 nm in diameter, properties approximately similar to those reported for the superior cervical ganglion of the newborn rat, with a vesicle diameter range of 90–150 nm (Eränkö, 1972) and those of the adult rat, with reported vesicle diameters of 80–100 nm (Hökfelt, 1969), 65–120 nm (Matthews and Raisman, 1969), 70–100 nm (Taxi, 1969) and average 140 nm (Williams and Palay, 1969). The size of the granular vesicles observed in SGC cells of control cultures in the present study is thus within the normal *in vivo* range. In addition to round vesicles similar to those in the SGC cells of living animals, elongated vesicles with an inner dense core of the same shape or with 2 separate granules, and hour-glass shaped vesicles with separate granules were a common feature in SGC cells in control cultures of the present study. Some spindle-shaped an irregular granules were reported in SGC cells of the superior cervical ganglion of the rat (Williams and Palay, 1969). Elongated granular vesicles were also reported by Watanabe (1971) in the hypogastric ganglion of the guinea pig and in the paracervical ganglion of the rat by Kanerva and Teräväinen (1972). This is in contrast to the observation that almost all granular vesicles are spherical in the normal superior cervical ganglion of the newborn rat (Eränkö, 1972) and in the sympathetic chain ganglia of the one-week-

old rat even after injections of hydrocortisone (Eränkö *et al.*, 1972b). Thus, the presence of elongated granular vesicles observed in the present study suggests that they have been formed in SGC cells under culture conditions.

Different shapes of granular vesicles, ranging from spherical to tubular and hour-glass shaped structures with either one or two dense granules, may indicate formation of new granular vesicles by central constriction and division of elongated vesicles. If this is so, the presence of numerous elongated granular vesicles may be a sign of an increased formation of spherical granular vesicles, and this would imply that the formation of granular vesicles is stimulated in at least some SGC cells under tissue culture conditions even when a normal control medium is used.

Examination of hydrocortisone-treated cultures by the fluorescence histochemical method for catecholamines showed an increase in the intensity of the fluorescence of these cells and a shift in the colour of the fluorescence (through the yellow filter K 510) from green to yellow, orange and red. These observations confirm those of the previous fluorescence microscopic study on cultures (Eränkö *et al.*, 1972), where it was concluded that there was an increased synthesis of catecholamines by the SIF cells *in vitro* and an increased rate of cell division and/or increased differentiation of SIF cells from less mature forms of sympathetic cells.

In the present electron microscopic examination of hydrocortisone-treated cultures of sympathetic ganglion explants, the number of SGC cells increased and also the number and size of granular vesicles in the cytoplasm of most of these cells increased relative to controls. There is little doubt that the SGC cells correspond to the SIF cells and that the increased fluorescence intensity observed in the SIF cells after culture in the hydrocortisone-containing medium is due to the increase both in the number and in the size of the granular vesicles in which the catecholamines are stored. Since dense material and granular vesicles were in the present study found in the Golgi vesicles, near the Golgi apparatus (see also Williams and Palay, 1969; Lever and Presley, 1971) and in peripheral tubular structures, especially in hydrocortisone-containing cultures, it is possible that hydrocortisone influences the function of these cell organelles.

Since the number of catecholamine-containing granular vesicles was increased in the SGC cells of the hydrocortisone-containing cultures, possibly similar increased formation of granular vesicles in less differentiated cells may lead to formationof new SGC or SIF cells. The results of the present study suggest that such a mechanism may be responsible for the increase in the number of these cells in the hydrocortisone-containing cultures. However, there may also have been mitotic divisions of pre-existing SGC cells. Mitotic division of the SGC cells has been electron microscopically observed in abdominal sympathetic paraganglia of 1–6 day old rats (Mascorro and Yates, 1970).

Four types of granular vesicles were observed in the present study: (1) round granular vesicles about 100 nm in diameter with a dense core; (2) elongated vesicles 40–150 × 150–250 nm with a dense core; (3) large round granular vesicles about 200 nm in diameter with a dense core and (4) large round granular vesicles about 200 nm in diameter with cores of low density. The first two types of granular vesicles were found both in the control cultures and those containing hydrocortisone, while the two latter types of larger size were observed in the hydrocortisone-

containing cultures only. Large granules about 200 nm in diameter have been previously reported *in vivo* in the inferior mesenteric ganglion of the rabbit (Elfvin, 1968) and the rat (Van Orden *et al.*, 1970), in the paracervical ganglion of the rat (Kanerva, 1972; Kanerva and Teräväinen, 1972) and, notably, in the hypogastric ganglion of the guinea pig, in which Watanabe (1971) described all the four types of granular vesicles mentioned above. It is interesting that the two types of large (200 nm) granular vesicles formed in hydrocortisone-containing cultures do also occur *in vivo*, in the prevertebral ganglia, although they are not found in the paravertebral sympathetic chain ganglia of rats.

As was emphasized by Watanabe (1971), the granular vesicles with cores of greater electron density resemble the noradrenaline-containing storage vesicles, while the vesicles with cores of lower electron density resemble the adrenaline-containing vesicles of the adrenal medulla (Coupland and Hopwood, 1966). This raises the question whether hydrocortisone causes the formation of adrenaline in addition to dopamine (Björklund *et al.*, 1970) and/or noradrenaline (Eränkö and Eränkö, 1971) which normally are contained in the granular vesicles. Hydrocortisone has previously been observed to cause a great increase in the number of extra-adrenal chromaffin cells of newborn rats and the intensity of their chromaffin reaction (Lempinen, 1964) and catecholamine fluorescence (Eränkö and Eränkö, 1972). Administration of hydrocortisone to newborn rats also causes the appearance of adrenaline in these cells, which normally contain exclusively noradrenaline (Eränkö *et al.*, 1966). Moreover, increase in the adrenaline content and replacement of highly electron-dense, noradrenaline-storing granular vesicles by vesicles with less dense content (presumably adrenaline-storing), has been observed in cortisone-treated cultures of extra-adrenal chromaffin cells of 2-day-old rabbits (Coupland and McDougall, 1966). It would seem possible that formation of adrenaline can occur in hydrocortisone-containing cultures of the SGC cells of the sympathetic ganglia, which morphologically and histochemically closely resemble the extra-adrenal chromaffin cells. This would be an interesting subject for further studies.

References

Björklund, A., Cegrell, L., Falck, B., Ritzen, M., Rosengren, E.: Dopamine-containing cells in sympathetic ganglia. Acta physiol. scand. **78**, 334–338 (1970).

Chamley, J.H., Mark, G.E., Burnstock, G.: Sympathetic ganglia in culture. II. Accessory cells. Z. Zellforsch. (in press) (1972b).

Chamley, J. H., Mark, G. E., Campbell, G. R., Burnstock, G.: Sympathetic ganglia in culture. I. Neurons. Z. Zellforsch. (in press) (1972a).

Coupland, R.E., Hopwood, D.: The mechanism of the differential staining reaction for adrenaline- and noradrenaline-storing granules in tissues fixed in glutaraldehyde. J. Anat. (Lond.) **100**, 227–243 (1966).

Coupland, R.E., MacDougall, J.D.B .: Adrenaline formation in noradrenaline-storing chromaffin cells *in vitro* induced by corticosterone. J. Endocr. **36**, 317–324 (1966).

Csillik, B., Kalman, G., Knyihar, E.: Adrenergic nerve endings in the feline cervicale superius ganglion. Experientia (Basel) **23**, 477–478 (1967).

Elfvin, L. G.: A new granule-containing nerve cell in the inferior mesenteric ganglion of the rabbit. J. Ultrastructure. Res. **22**, 37–44 (1968).

Eränkö, L.: Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. Brain Res. (in press) 1972.

Eränkö, L., Eränkö, O.: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. Acta physiol. scand. **84**, 125–133 (1972).

Eränkö, O.: The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. J. roy. micr. Soc. **87**, 259–276 (1967).

Eränkö, O., Eränkö, L.: Small intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. Progr. Brain Res. **34**, 39–51 (1971).

Eränkö, O., Eränkö, L., Hill, C. E., Burnstock, G.: Hydrocortisone-induced increase in the number of small intensely fluorescent cells and their histochemically demonstrable catecholamine content in cultures of sympathetic ganglia of the newborn rat. Histochem. J. **4**, 49–58 (1972a).

Eränkö, O., Härkönen, M.: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. Acta physiol. scand. **58**, 285–286 (1963).

Eränkö, O., Härkönen, M.: Effect of axon division on the distribution of noradrenaline and acetylcholinesterase in sympathetic neurons of the rat. Acta physiol. scand. **63**, 411–412 (1965a).

Eränkö, O., Härkönen, M.: Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. Acta physiol. scand. **63**, 511–512 (1965b).

Eränkö, O., Heath, J. W., Eränkö, L.: Effect of hydrocortisone on the ultrastructure of the small, granule-containing cells in the superior cervical ganglion of the newborn rat. (In the course of publication) (1972b).

Eränkö, O., Lempinen, M., Räisänen, L.: Adrenaline and noradrenaline in the organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. Acta physiol. scand. **66** 253–254 (1966).

Grillo, M. A.: Electron microscopy of sympathetic tissues. Pharmacol. Rev. **18**, 387–399 (1966).

Hökfelt, T.: Distribution of noradrenaline storing particles in peripheral adrenergic neurons as revealed by electron microscopy. Acta physiol. scand. **76**, 427–440 (1969).

Jacobowitz, D.: Histochemical studies of the relationship of chromaffin cells and adrenergic nerve fibres of the cardiac ganglia of several species. J. Pharmacol. exp. Ther. **158**, 227–240 (1967).

Jacobowitz, D.: Catecholamine fluorescence studies of adrenergic neurons and chromaffin cells in sympathetic ganglia. Fed. Proc. **29**, 6, 1929–1944 (1970).

Kanerva, L.: The postnatal development of monoamines and cholinesterase in the paracervical ganglion of the rat uterus. Progr. Brain Res. **34**, 433–444 (1971).

Kanerva, L.: Ultrastructure of sympathetic ganglion cells and granule-containing cells in the paracervical (Frankenhäuser) ganglion of the newborn rat. Z. Zellforsch. **126**, 25–40 (1972).

Kanerva, L., Täräväinen, H.: Electron microscopy of the paracervical (Frankenhäuser) ganglion of the adult rat. Z. Zellforsch. **129**, 161–177 (1972).

Lempinen, M.: Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. Acta physiol. scand. **62**, Suppl. 231 (1964).

Lever, J. D., Presley, R.: Studies on the sympathetic neurone *in vitro*. Progr. Brain Res. **34**, 499–512 (1971).

Mascorro, J. A., Yates, R. D.: Microscopic observations in abdominal sympathetic paraganglia. Tex. Rep. Biol. Med. **28**, 59–68 (1970).

Matthews, M. R., Raisman, G.: The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. J. Anat. (Lond.) **105**, 255–282 (1969).

Norberg, K. A., Hamberger, B.: The sympathetic adrenergic neuron. Acta physiol. scand. **63**, Suppl. 238 (1964).

Norberg, K. A., Ritzén, M., Ungerstedt, U.: Histochemical studies on a special catecholamine-containing cell type in sympathetic ganglia. Acta physiol. scand. **67**, 260–270 (1966).

Norberg, K. A., Sjöqvist, F.: New possibilities for adrenergic modulation of ganglionic transmission. Pharmacol. Rev. **18**, 743–751 (1966).

Olson, L.: Outgrowth of sympathetic adrenergic neurons in mice treated with a nerve growth factor (NGF). Z. Zellforsch. **81**, 155–173 (1967).

Orden, L. S. III van, Burke, J. P., Geyer, M., Lodoen, F. V.: Localization of depletion-sensitive and depletion-resistant norepinephrine sites in autonomic ganglia. J. Pharmacol. exp. Ther. **174**, 56–71 (1970).

Owman, C., Sjöstrand, N.-O.: Short adrenergic neurons and catecholamine containing cells in vas deferens and accessory male genital glands of different mammals. Z. Zellforsch. **66** 300–320 (1965).

Rose, G. C.: A separable and multipurpose tissue culture chamber. Tex. Rep. Biol. Med. **12**, 1074–1083 (1954).

Salk, E. S., Youngner, J. S., Ward, E. N.: Use of color change of phenol red as the indicator in titrating poliomyelitis virus on its antibody in a tissue culture system. Appendix. Method of preparing medium 199. Amer. J. Hyg. **60**, 214–230 (1954).

Siegrist, G., Dolivo, M., Dunant, Y., Foroglou-Kerameus, C., Ribaupierre, F. de., Rouiller, C.: Ultrastructure and function of the chromaffin cells in the superior cervical ganglion of the rat. J. Ultrastruct. Res. **25**, 381–407 (1968).

Taxi, J., Gautron, J., L'Hermite, P.: Données ultrastructurales sur une éventuelle modulation adrénergique de l'activé du ganglion cervical superieur du Rat. C. R. Acad. Sci. (Paris) **269**, 1281–1284 (1969).

Watanabe, H.: Adrenergic nerve endings in the peripheral autonomic ganglion. Experientia (Basel) **26**, 69–70 (1970).

Watanabe, H.: Adrenergic nerve elements in the hypogastric ganglion of the guinea-pig. Amer. J. Anat. **130**, 305–330 (1971).

Williams, T. H.: Electron microscopic evidence for an autonomic interneuron. Nature (Lond.), **214**, 39–310 (1967).

Williams, T. H., Palay, S. L.: Ultrastructure of the small neurons in the superior cervical ganglion. Brain Res. **15**, 17–34 (1969).

Professor Dr. O. Eränkö
Anatomisches Institut der Universität
Siltavuorenpenger 20b A
Helsinki 17, Finnland 00170

Separatum EXPERIENTIA *29*, 457 (1973)
Birkhäuser Verlag, Basel (Schweiz)

Effect of Hydrocortisone on the Ultrastructure of the Small, Granule-Containing Cells in the Superior Cervical Ganglion of the Newborn Rat

Small cells with an intense formaldehyde-induced catecholamine fluorescence are present amongst sympathetic nerve cells in the superior cervical ganglion of adult[1,2] and newborn[3] rats. Electron microscopic studies have shown that these cells contain round granular vesicles, about 100 nm in diameter, in the ganglia of both adult[4] and newborn[5] rats. Administration of hydrocortisone has been shown to cause a 10-fold increase in the number of the small, intensely fluorescent cells in the sympathetic ganglia of newborn, but not adult, rats[6]. The present study was undertaken to investigate the ultrastructure of such newly formed catecholamine-containing cells.

Twelve newborn rats of the Sprague-Dawley strain were i.p. injected with 20 mg/kg body weight of hydrocortisone acetate daily for 7 days. They were killed 1 day after the last injection, together with untreated controls of the same age. The superior cervical ganglia were removed and processed for electron microscopy using a procedure generally found useful in the present laboratory[7]. Following a brief initial fixation in 1% osmium tetroxide in 0.1 *M* phosphate buffer (pH 7.4), the tissue was diced and replaced in the same fluid for 30 min; it was then washed in the buffer for 10 min and post-fixed in buffered 5% glutaraldehyde for 30 min. After a 10 min buffer wash the tissue was replaced in buffered 1% osmium tetroxide for 30 min. Following a short rinse in distilled water, it was block-stained in aqueous saturated uranyl acetate for 1 h, dehydrated in a graded acetone series, infiltrated in a mixture of equal volumes of acetone and Araldite, and finally embedded in Araldite. Thin sections were cut with a Huxley-Cambridge ultramicrotome, double-stained with a satured aqueous solution of uranyl acetate followed by lead citrate[8] and subsequently examined with a Hitachi 11 B electron microscope.

Small, granule-containing cells with typical appearance[4,5] were observed in the control ganglia. In the ganglia of the hydrocortisone-treated rats, the small granule-containing cells were much more numerous; a cluster of such cells in the ganglion of a hydrocortisone-injected rat is illustrated in Figure 1. Round vesicles with a dense core

[1] O. Eränkö and M. Härkönen, Acta physiol. scand. *58*, 285 (1963).
[2] O. Eränkö and M. Härkönen, Acta physiol. scand. *63*, 511 (1965).
[3] O. Eränkö and L. Eränkö, Progr. Brain Res. *34*, 39 (1971).
[4] M. R. Matthews and G. Raisman, J. Anat. *105*, 255 (1969).
[5] L. Eränkö, Brain Res. *46*, 159 (1972).
[6] L. Eränkö and O. Eränkö, Acta physiol. scand. *84*, 125 (1972).
[7] J. W. Heath, B. K. Evans, B. J. Gannon, G. Burnstock and V. B. James, Virchows Arch. Abt. B. Zellpath., *11*, 182 (1972).
[8] E. S. Reynolds, J. Cell Biol. *17*, 208 (1963).

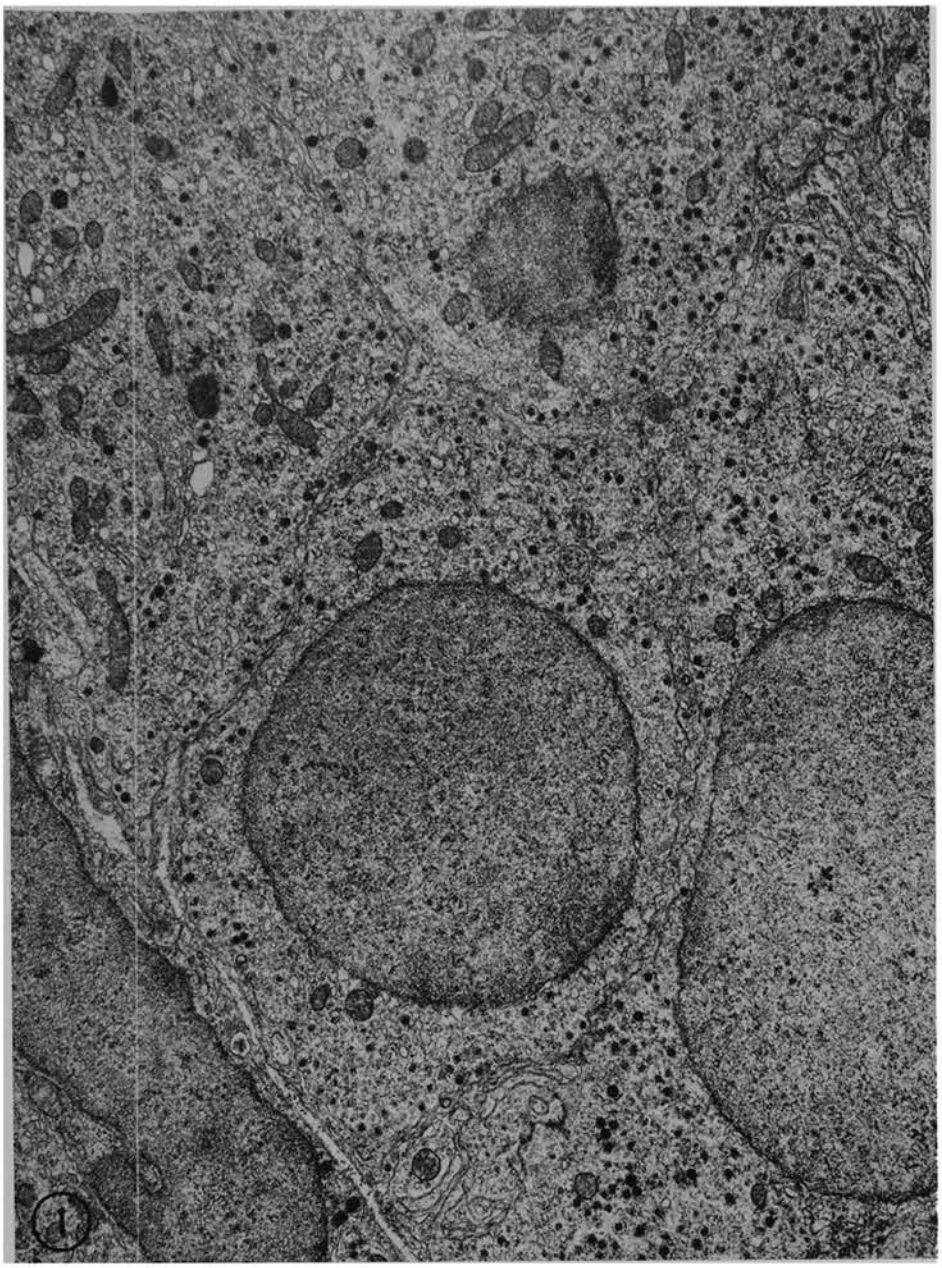


Fig. 1. Low power photomicrograph of part of a cluster of small granule-containing cells from a hydrocortisone-treated rat given 20 mg/kg hydrocortisone acetate daily for 7 days. × 13,000.

were seen throughout the cytoplasm, their number being increased as compared with those in the small granule-containing cells of untreated rats. The diameter of the granular vesicles was about 100 nm both in the control and in the hydrocortisone-treated rats (Figure 2a, b). The electron density of the contents of the vesicles ranged from low to high in both control and hydrocortisone-treated rats, but vesicles with cores of high density were much more numerous in the small granule-containing cells of the treated group (Figure 2b, cf. a).

The observation of the present study that hydrocortisone causes an increase, both in the number of the small granule-containing cells and in the number of the granular vesicles in them, strongly suggests that catecholamines in the newly formed cells are mainly stored in the granular vesicles (as in similar cells of normal rats), rather than in the cytoplasm outside them, an alternative considered earlier[6]. This is in agreement with the observations made in a recent study on the effect of hydrocortisone on cultures of newborn rat sympathetic ganglia, in which an increase in the number of the small, intensely fluorescent cells and in the intensity of their fluorescence was paralleled by an increase in the number of small granule-containing cells and an increase in the number of granular vesicles in them[9].

The significance of the variation in the electron density observed in the present study between individual granular vesicles of the hydrocortisone-injected rats may reflect either variation in the amine concentration of the central granule or differences in its amine composition. This matter should be further studied because the histochemical significance of the method of fixation employed in the present study (osmium-glutaraldehyde-osmium) has not been established. However, it is of interest that, in

[9] O. ERÄNKÖ, J. W. HEATH and L. ERÄNKÖ, Z. Zellforsch, *134*, 297 (1972).

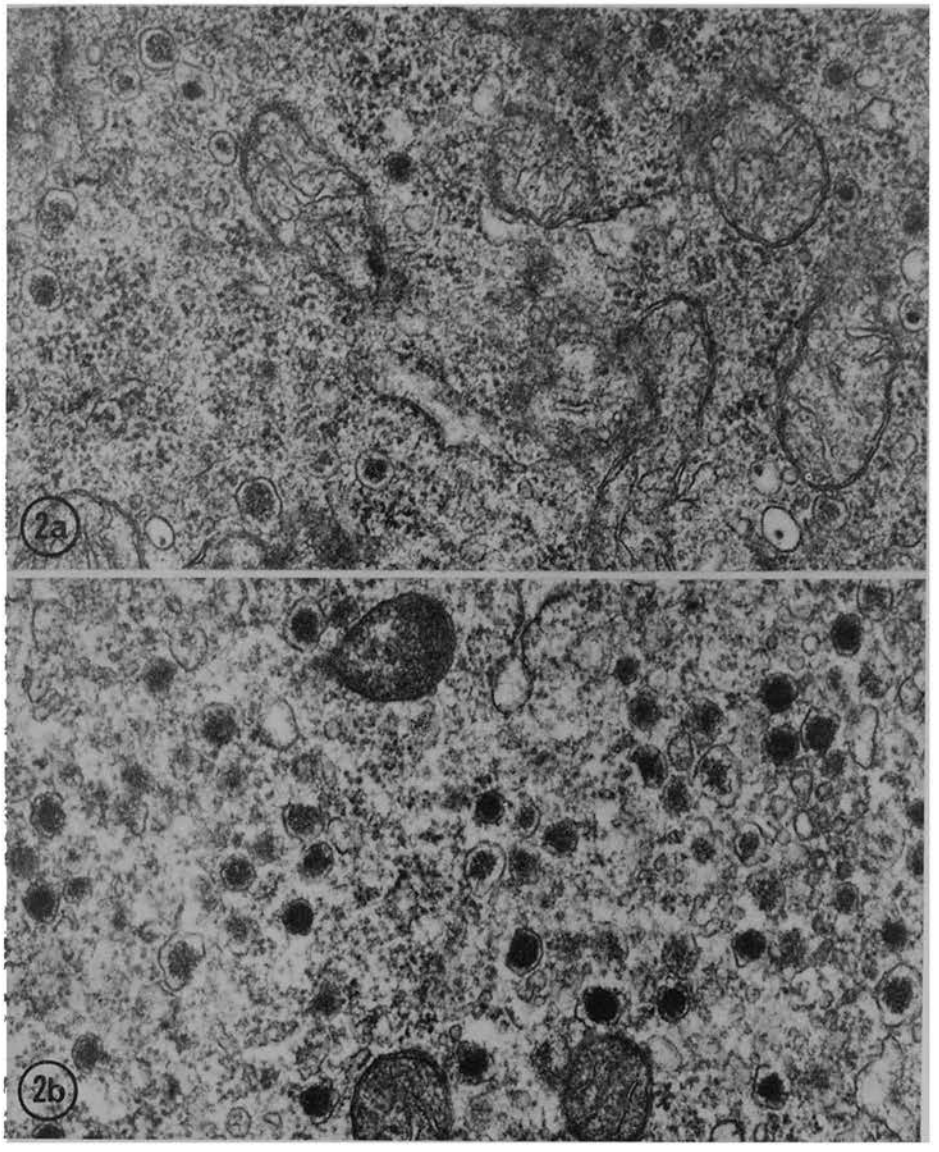


Fig. 2. Areas of the cytoplasm of small granule containing cells. a) Control rat. The diameter of the granular vesicles is about 100 nm. The cores of the granular vesicles vary in electron density from low to high, but most cores are of low density. × 50,000. b) Hydrocortisone treated rat. The diameter of granular vesicles and the range in electron density of their cores are similar to that of controls but most granular vesicles have cores of high density; 20 mg/kg/day hydrocortisone acetate for 7 days. × 50,000.

material fixed first in glutaraldehyde and then in osmium, the adrenaline-containing vesicles contain cores of low electron density and the noradrenaline-containing vesicles contain cores of high electron density[10,11]. Granules of both low and high density have been observed in the small granule-containing cells in hydrocortisone-containing cultures after glutaraldehyde-osmium fixation, while only highly dense granules were seen in control cultures[9]. Previous chemical observations have shown that hydrocortisone causes the appearance of adrenaline in the extra-adrenal chromaffin cells, which normally contain only noradrenaline, both in vivo[12] and in vitro[13], suggesting that hydrocortisone may cause the formation of adrenaline also in the small granule-containing cells of the sympathetic ganglia. However, the increase in electron density of the granular vesicles of the small granule-containing cells of hydrocortisone-treated rats observed in the present study using primary fixation in osmium tetroxide more probably reflects an increase in the amine concentration in the vesicles[14].

Zusammenfassung. Hydrocortison-Injektionen führen bei neugeborenen Ratten zu einer Vermehrung der kleinen granulahaltigen Zellen im Ganglion cervicale craniale und zu einer Vermehrung der granulären Vesikeln im Zytoplasma dieser Zellen.

O. Eränkö[15], J. W. Heath[16] and L. Eränkö[17]

Department of Zoology, University of Melbourne, Parkville, (Victoria Australia 3052), 12 October 1972.

[10] R. E. Coupland and D. Hopwood, J. Anat. *100*, 227 (1966).
[11] H. Watanabe, Am. J. Anat. *130*, 305 (1971).
[12] O. Eränkö, M. Lempinen and L. Räisänen, Acta physiol. scand. *66*, 253 (1966).
[13] R. E. Coupland and J. D. B. MacDougall, J. Endocrin. *36*, 317 (1966).
[14] This study was supported by the National Health Foundation of Australia, the Australian Research Grants Committee and the Sigfrid Jusélius Foundation, Finland.
[15] University of Melbourne Senior Research Fellow September 1971–August 1972. Present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland 00170.
[16] In receipt of a grant from the National Health and Medical Research Council of Australia.
[17] Sunshine Foundation and Rowden White Overseas Research Fellow September 1971–August 1972. Present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland 00170.

Acta pharmacol. et toxicol. 1973, **33**, 209–218.

From the Department of Zoology, University of Melbourne, Parkville, Victoria, Australia, 3052

Effect of Guanethidine on the Ultrastructure of the Small, Granule-containing Cells in Cultures of Rat Sympathetic Ganglia[1]

By

John Heath[2], Olavi Eränkö[3] and Liisa Eränkö[4]

(Received November 28, 1972; Accepted January 5, 1973)

Abstract: Sympathetic chain ganglia of newborn rats were cultured in Rose chambers with and without guanethidine. After one week, the cultures were examined by light microscopy for formaldehyde-induced catecholamine fluorescence and by electron microscopy after fixation in 5 % glutaraldehyde and 1 % osmium tetroxide. Guanethidine sulphate (2 mg/l) caused an increase in the number of the small, intensely fluorescent (SIF) cells in the ganglion explants. Electron microscopic examination of guanethidine-containing cultures revealed an increased number of small, granule-containing (SGC) cells, which corresponded in size and shape to the SIF cells. Round vesicles (about 100 nm in diameter) and elongated vesicles (about 80 nm in cross section and about 200 nm in length) containing an electron-dense core were observed in the cytoplasm of the SGC cells both in control and guanethidine-containing cultures. The granular vesicles were most frequent in the periphery of the cytoplasm. In ganglia cultured with guanethidine, most SGC cells observed contained a greatly reduced number of granular vesicles as compared to SGC cells of the control cultures.

Key-words: Guanethidine – small granule-containing cells – cultures – ultrastructure.

---

[1] This work was supported by the National Heart Foundation, the Australian Research Grants Committee and the Sigrid Juselius Foundation.

[2] In receipt of a grant from the National Health and Medical Research Council of Australia.

[3] University of Melbourne Senior Research Fellow, September 1971 – August 1972; present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland 00170.

[4] Sunshine Foundation and Rowden White Overseas Research Fellow in the University of Melbourne, September 1971 – August 1972; present address: Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland 00170.

Sympathetic ganglia are known to contain small, intensely fluorescent (SIF) cells amongst typical sympathetic nerve cells (ERÄNKÖ & HÄRKÖNEN 1963 & 1965; review by ERÄNKÖ & ERÄNKÖ 1971b). These cells correspond electronmicroscopically to small, granule-containing (SGC) cells, which are characterised chiefly by the presence of cytoplasmic vesicles containing an electron-dense core (MATTHEWS & RAISMAN 1969).

Prolonged administration of guanethidine has been shown to cause degenerative changes in neurons of the sympathetic ganglia of adult rats (JENSEN-HOLM & JUUL 1970 & 1971; BURNSTOCK *et al.* 1971; HEATH *et al.* 1972; ERÄNKÖ & ERÄNKÖ 1971b). In newborn rats, guanethidine causes destruction of sympathetic nerve cells (ERÄNKÖ & ERÄNKÖ 1971a; ANGELETTI *et al.* 1972) but a marked increase in the number of the SIF cells (ERÄNKÖ & ERÄNKÖ 1971a). A similar increase in the number of SIF cells has been observed in cultures of sympathetic ganglia containing a low or moderate concentration of guanethidine (ERÄNKÖ *et al.* 1972a). The present study was undertaken to compare the ultrastructure of these cells in control cultures and in cultures containing guanethidine.

Materials and methods

Newborn albino rats of the Sprague-Dawley strain were killed by a blow on the head. The thoracic and abdominal sympathetic chain was dissected with the aid of a binocular microscope under sterile conditions, carefully avoiding damage to the ganglia. The chains were then treated with 0.125 % trypsin in a balanced salt solution, and after cutting the connections the separated ganglia were transferred to collagen-coated coverslips.

Details of the method of tissue culture have been described by CHAMLEY *et al.* (1972). However, it should be mentioned here that the cultures were carried out in modified ROSE (1954) chambers containing Medium 199 (SALK *et al.* 1954) supplemented with 20 % (v/v) of foetal calf serum, 0.05 units/ml of insulin, 100 units/ml of penicillin G, 5 mg/ml of glucose and 1 unit/ml of nerve growth factor (Burroughs and Wellcome, England). Guanethidine sulphate (ismelin®, Ciba-Geigy, Basle) was dissolved in the balanced salt solution to make a stock solution containing 0.2 mg/ml. This was filtered through a millipore filter and added to the culture medium in a proportion of 1:99, thus obtaining a final concentration of 2 micrograms of guanethidine sulphate per ml culture medium or 2 mg/l. The culture chambers were kept in an incubator at 37° supplied with a continuous flow of 5 % carbon dioxide in atmospheric air (Commonwealth Industrial Gases, Australia) bubbled through water. Both guanethidine-containing and control culture media were changed daily.

The material comprised 12 guanethidine-containing chambers and 12 control chambers, each containing 4 explants. The cultures were carried out for 7 days. They were opened on the 8th day, and 4 control and 4 guanethidine-containing chambers were prepared for fluorescence microscopy. The remaining 8 control and 8 guanethidine-containing chambers were prepared for electron microscopy, and one explant from each of these was selected for detailed analysis.

For fluorescence microscopic demonstration of catecholamines the cultures were first dried on the coverslip overnight in a dessicator containing phosphorus pentoxide at room temperature. They were then exposed to formaldehyde vapour from paraformaldehyde powder, equilibrated with air of 60 % relative humidity in loosely closed jars at 50° for 30 min. and subsequently at 80° for 1 hour. They were then mounted in liquid paraffin and examined in a Leitz Ortholux fluorescence microscope with an HBO 200 mercury lamp and a filter combination of BG12 and K510. Further details on the histochemical method are given in previous papers (ERÄNKÖ 1967; ERÄNKÖ *et al.* 1972b).

Material was prepared for electron microscopy by successive immersion in the following solutions at room temperature: Cultures on the coverslips were fixed in 5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min., washed in buffer for 10 min., post-fixed in 1 % buffered osmium tetroxide for 30 min., briefly rinsed in distilled water, stained in 2 % aqueous solution of uranyl acetate for 1 hour, dehydrated in a graded acetone series, infiltrated in a mixture of equal parts of acetone and Araldite and finally embedded in Araldite. Thin sections cut with a Huxley-Cambridge ultramicrotome were double stained with a saturated aqueous solution of uranyl acetate followed by lead citrate (REYNOLDS 1963) and were subsequently examined with an Hitachi 11B electron microscope.

Results

Catecholamine fluorescence.

Control cultures. SIF cells in the control cultures were usually found in small clusters, and exhibited a green fluorescence of low to moderate intensity. Some single SIF cells were also seen. Other areas of the ganglion explants exhibited only a weak diffuse fluorescence forming a background against which the SIF cell clusters stood out clearly.

Guanethidine-containing cultures. In guanethidine-containing cultures the clusters of SIF cells were larger and more numerous. The colour of the fluorescence was green as in the control cultures, and the fluorescence intensity ranged from weak to moderate. However, compared to control cultures, a greater number of weakly fluorescent SIF cells was observed.

Ultrastructure.

Control cultures. The nuclei of SGC cells in control cultures showed dense chromatin aggregation, and the cytoplasm contained numerous mitochondria and abundant free ribosomes, usually in rosette formation. Cisternae of rough endoplasmic reticulum and microtubules were present. The granular vesicles, typically abundant in these cells, were mostly located in the periphery of the cytoplasm (fig. 1). The granular vesicles were round (50–150 nm in diameter) or elongated (50–100 nm in width and 150–250 nm in length) in profile. The central granule was usually of the same shape as the vesicle surrounding it, and an electron-lucent halo, usually 15–20 nm in width,

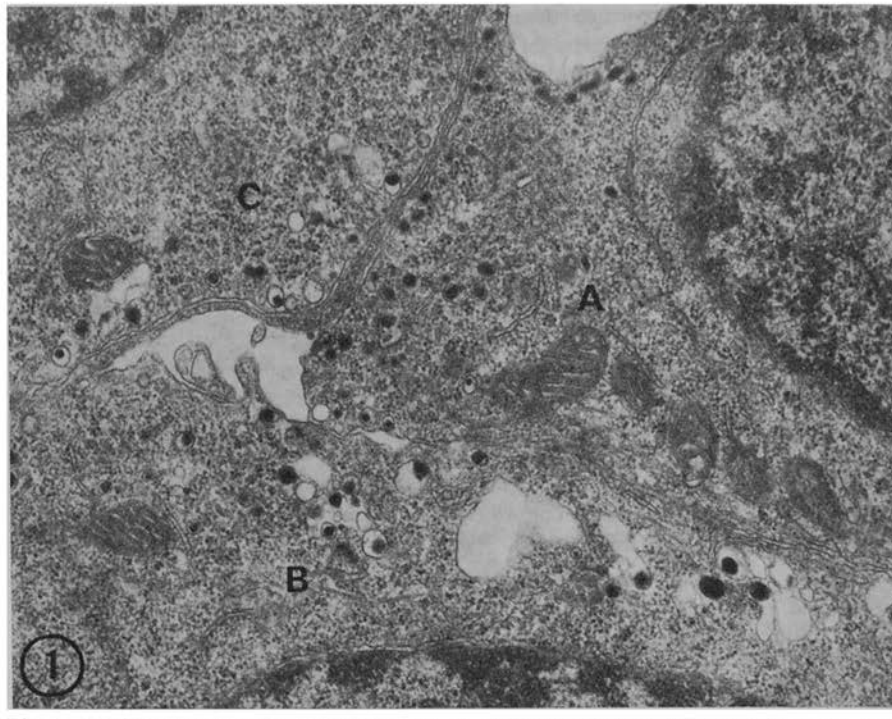


Fig. 1. Typical appearance of SGC cells in control cultures. The nuclei show dense chromatin aggregation, particularly in the periphery, and the cytoplasm contains an abundance of organelles. The granular vesicles are located mainly in the periphery of the cytoplasm. The cell membranes of cell A and cell B are in close contact while cell A and cell C are separated by a thin satellite cell process. 7 day culture. Magnification × 22,000.

was present between the granule and the vesicle membrane (fig. 3). In some areas the outer cell membranes of the SGC cells were in close contact while in others a thin layer of satellite cell cytoplasm was observed between the SGC cells (fig. 1).

Guanethidine-containing cultures. The number of SGC cells observed was distinctly increased in all the guanethidine-containing cultures examined. Although some variation existed between individual cultures, in approximately 75 % of observed SGC cells the population of granular vesicles was markedly reduced as compared to SGC cells of control cultures (fig. 2). Many SGC cells in guanethidine-containing cultures contained only 10–20 granular vesicles per complete cell profile, compared to 100–250 granular vesicles per complete profile of a typical SGC cell of similar size in control cultures. The mitochondria in some SGC cells in guanethidine-con-

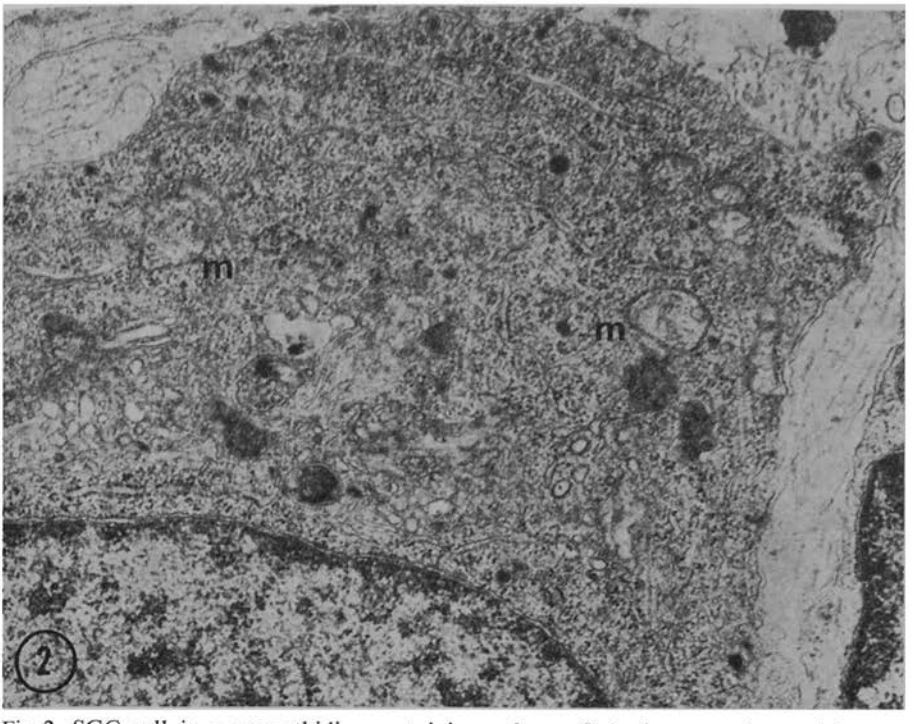


Fig. 2. SGC cell in a guanethidine-containing culture. Only few granular vesicles are present in the cytoplasm, and the mitochondria (m) show damaged cristae and decreased density of the matrix. Otherwise the structure of the cell is similar to controls. 7 day culture with 2 mg/l guanethidine sulphate included in the culture medium. Magnification × 22,000.

taining cultures (particularly those with greatest reduction of granular vesicles) showed structural damage to cristae, decreased density of the matrix and also swelling in some instances. The ultrastructure of this group of SGC cells appeared to be otherwise normal. Some SGC cells (approximately 25 %) observed in guanethidine-containing cultures, however, appeared to be similar to typical SGC cells of control cultures in all ultrastructural aspects, including the population of granular vesicles (fig. 4). The mitochondria of SGC cells in this minority group were similar in size to those in control SGC cells, and were always structurally intact. It should be stated that a small number of observed SGC cells contained a number of granular vesicles intermediate between the two extremes described above, giving the impression that a continuum existed from the SGC cells with only few granules to those with numbers typical of SGC cells in control cultures.

The dimensions and shapes of the granular vesicles in SGC cells of guan-

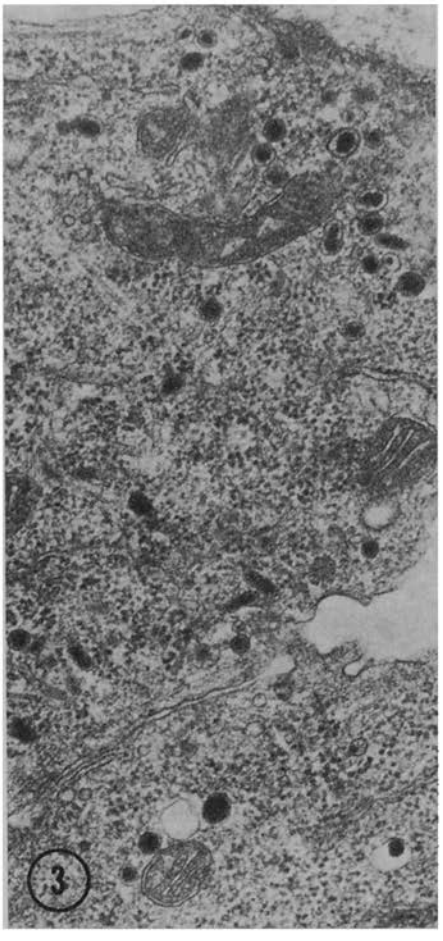


Fig. 3. Control culture. The granular vesicles of the SGC cell are round or elongated. The granule takes the same shape as the bounding membrane. An electron-lucent halo is present between the membrane of the vesicle and its core. 7 day culture. Magnification × 35,000.

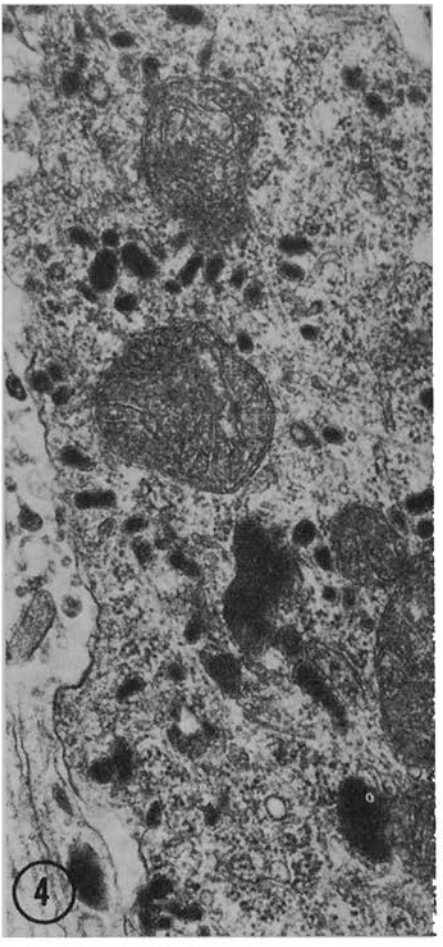


Fig. 4. Peripheral region of an SGC cell from a guanethidine-containing culture. The number of granular vesicles in this cell is comparable with that in SGC cells of control cultures. The granular vesicles are of similar dimensions to those in control cultures and the cores of the vesicles are of similar electron-density to the controls (cf. fig. 3). Note that the mitochondria are structurally intact. (The large size of some of the mitochondria in this micrograph represents only an occaisional finding in this specimen). 7 day culture with 2 mg/l guanethidine sulphate included in the culture medium. Magnification × 35,000.

ethidine-containing cultures were similar to those of control cultures, and there was no significant tendency towards either an increase or decrease in the size or electron density of the central core in SGC cells of the guanethidine-treated cultures (fig. 3, cf. 4). Depleted or "empty" vesicles were not observed in SGC cells of guanethidine-treated cultures.

Nerve cells and satellite cells appeared normal both in the control and the guanethidine-containing cultures.

Discussion

The increase in the number of SIF cells observed in the present study after the addition of 2 mg/l of guanethidine sulphate to the culture medium is in agreement with the previous observations that 1 and 3 mg/l of the same drug causes an increase in the number of SIF cells in cultures (ERÄNKÖ *et al.* 1972a) and that guanethidine injections given to newborn rats result in a hyperplasia of these cells (ERÄNKÖ & ERÄNKÖ 1971a). The increase in the number of the SGC cells observed by electron microscopy in the present study is consistent with this increased number in the SIF cell population, since the SIF cells are believed to correspond to the SGC cells not only in sympathetic ganglia of normal adult (MATTHEWS & RAISMAN 1969; VAN ORDEN *et al.* 1970; ERÄNKÖ & ERÄNKÖ 1971b) and newborn (ERÄNKÖ 1972) rats but also in cultures of sympathetic ganglia (LEVER & PRESLEY 1971; ERÄNKÖ *et al.* 1972c).

The observation that the number of granular vesicles in most SGC cells of the guanethidine-containing cultures was less than that in SGC cells of controls is interesting, especially in view of the previous observation that an increase in the number of SGC cells during hydrocortisone treatment was associated with an *increase* in the number of granular vesicles in those cells both *in vitro* (ERÄNKÖ *et al.* 1972c) and *in vivo* (ERÄNKÖ *et al.* 1973). This suggests that the mechanisms by which hydrocortisone and guanethidine cause an increase in the number of SGC cells may be at least partly different. It is possible that guanethidine may stimulate mitotic division of the SGC cells; this mechanism has been previously proposed following the observation that in the sympathetic ganglia of newborn rats injected with guanethidine the number of SIF cells in each cluster was greatly increased while the number of clusters was essentially unaffected (ERÄNKÖ & ERÄNKÖ 1971a). Electron microscopic observation of mitotic division of SGC cells in newborn rats has been reported by MASCORRO & YATES (1970). Although mitoses were not observed in the present study, these events could have occurred prior to the time of examination (after culture for 7 days). Increased formation of catecholamine-containing granular vesicles in primitive cells, presumably pre-

cursors of the SIF or SGC cells, has been proposed as a possible additional explanation for the apperance of numerous new SIF or SGC cells in newborn rats (ERÄNKÖ & ERÄNKÖ 1972; ERÄNKÖ *et al.* 1973) and in cultures of sympathetic ganglia (ERÄNKÖ *et al.* 1972b & 1972c) treated with hydrocortisone. However, in the present study, this explanation would seem less likely since the fluorescence intensity and number of granular vesicles were decreased in many SIF or SGC cells. That guanethidine causes at the same time an increase in the number of SGC cells and a loss in the average number of granular vesicles in them may be due to two separate actions of this drug, i. e., stimulation of mitotic division of these cells and depletion of catecholamines from their storage granules. Such mechanisms may explain the very variable reactions of the SIF cells observed in cultures containing high concentrations of guanethidine (ERÄNKÖ *et al.* 1972a).

Studies of chronic high dose guanethidine treatment *in vivo* have shown that the mitochondria of adrenergic neurons, which selectively take up guanethidine, represent a primary site of action of this drug (JENSEN-HOLM & JUUL 1971; HEATH *et al.* 1972). Thus the mitochondrial damage observed in some SGC cells of guanethidine-containing cultures may represent a direct effect of this drug. In this regard it is interesting that the SGC cells showing mitochondrial damage were those with the greatest reduction in the number of granular vesicles. Since the concentration of guanethidine surrounding the ganglia in the present *in vitro* study is likely to be low compared to those which elicited damage in the *in vivo* studies (where doses of 10–40 mg/kg (JENSEN-HOLM & JUUL 1971) and 30–100 mg/kg (HEATH *et al.* 1972) were employed), this may explain why no ultrastructural sign of toxic effect was observed in the sympathetic neurons.

REFERENCES

Angeletti, P. U., R. Levi-Montalcini & F. Caramia: Structural and ultrastructural changes in developing sympathetic ganglia induced by guanethidine. *Brain Research* 1972, **43**, 515—525.

Burnstock, G., B. Evans, B. J. Gannon, J. W. Heath & V. James: A new method of destroying adrenergic nerves in adult animals using guanethidine. *Brit. J. Pharmacol.* 1971, **43**, 295–301.

Chamley, J. H., G. E. Mark, G. R. Campbell & G. Burnstock: Sympathetic ganglia in culture. I. Neurons. *Z. Zellforsch.* 1972, **135**, 287–314.

Eränkö, L.: Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. *Brain Research* 1972, **46**, 159–175.

Eränkö, L. & O. Eränkö: Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats. *Acta pharmacol. et toxicol.* 1971a, **30**, 403–416.

Eränkö, L. & O. Eränkö: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. *Acta physiol. scand.* 1972, **84**, 125–133.

Eränkö, L., C. E. Hill, O. Eränkö & G. Burnstock: Lack of toxic effect of guanethidine in nerve cells and small intensely fluorescent cells in cultures of sympathetic ganglia of newborn rats. *Brain Research* 1972a, **43**, 501–513.

Eränkö, O.: The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. roy. micr. Soc.* 1967, **87**, 259–276.

Eränkö, O. & L. Eränkö: Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Progr. Brain Research* 1971b, **34**, 39–51.

Eränkö, O., L. Eränkö, C. E. Hill & G. Burnstock: Hydrocortisone-induced increase in the number of small intensely fluorescent cells and their histochemically demonstrable catecholamine content in cultures of sympathetic ganglia of the newborn rat. *Histochem. J.* 1972b, **4**, 49–58.

Eränkö, O. & M. Härkönen: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* 1963, **58**, 285–286.

Eränkö, O. & M. Härkönen: Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* 1965, **63**, 511–512.

Eränkö, O., J. W. Heath & L. Eränkö: Effect of hydrocortisone on the ultrastructure of the small, intensely fluorescent, granule-containing cells in cultures of sympathetic ganglia of newborn rats. *Z. Zellforsch.* 1972c, **134**, 297–310.

Eränkö, O., J. W. Heath & L. Eränkö: Effect of hydrocortisone on the ultrastructure of the small granule-containing cells in the superior cervical ganglion of the newborn rat. *Experientia* 1973, in press.

Heath, J. W., B. K. Evans, B. J. Gannon, G. Burnstock & V. B. James: Degeneration of adrenergic neurons following guanethidine treatment: an ultrastructural study. *Virchows Arch. Abt. B. Zellpath.* 1972, **11**, 182–197.

Jensen-Holm, J. & P. Juul: The effects of guanethidine, pre- and post-ganglionic nerve division on the rat superior cervical ganglion: cholinesterases and catecholamines (histochemistry), and histology. *Acta pharmacol. et toxicol.* 1970, **28**, 283–298.

Jensen-Holm, J. & P. Juul: Ultrastructural changes in rat superior cervical ganglion following prolonged guanethidine administration. *Acta pharmacol. et toxicol.* 1971, **30**, 308–320.

Lever, J. D. & R. Presley: Studies on the sympathetic neurone *in vitro*. *Progr. Brain Research* 1971, **34**, 499–512.

Mascorro, J. A. & R. D. Yates: Microscopic observations in abdominal, sympathetic paraganglia. *Texas Rep. Biol. Med.* 1970, **28**, 59–68.

Matthews, M. R. & G. Raisman: The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. *J. Anat.* 1969, **105**, 255–282.

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 1963, **17**, 208–212.

Rose, G. C.: A separable and multipurpose tissue culture chamber. *Tex. Rep. Biol. Med.* 1954, **12**, 1074–1083.

Salk, E. S., J. S. Youngner & E. N. Ward: Use of colour change of phenol red as the indicator in titrating poliomyelitis virus on its antibody in tissue culture system. Appendix. Method of preparing medium 199. *Amer. J. Hyg.* 1954, **60**, 214–230.

Van Orden, L. S. III, J. P. Burke, M. Geyer & F. V. Lodoen: Localization of depletion-sensitive and depletion-resistant norepinephrine sites in autonomic ganglia. *J. Pharmacol. exp. Therap.* 1970, **174**, 56–71.

UROPEAN JOURNAL OF PHARMACOLOGY 23 (1973) 162–174. NORTH-HOLLAND PUBLISHING COMPANY

USE OF TISSUE CULTURE TO EXAMINE THE ACTIONS OF GUANETHIDINE AND 6-HYDROXYDOPAMINE[1]

Caryl E. HILL[2], Gerda E. MARK, Olavi ERÄNKÖ[3], Liisa ERÄNKÖ[4] and Geoffrey BURNSTOCK

Department of Zoology, University of Melbourne, Parkville, 3052, Victoria, Australia

Received 30 October 1972 Accepted 1 March 1973

C.E. HILL, G.E. MARK, O. ERÄNKÖ, L. ERÄNKÖ and G. BURNSTOCK, *Use of tissue culture to examine the actions of guanethidine and 6-hydroxydopamine*, European J. Pharmacol. 23 (1973) 162–174.

Cultures of newborn rat and embryo chick sympathetic ganglia have been used to compare the effects of 6-hydroxydopamine and guanethidine in vitro. In sufficient concentrations both drugs had a direct cytotoxic effect on the sympathetic neurons, eventually resulting in cell death. The major difference between the actions of the two drugs was that 6-hydroxydopamine caused nerve fibre fragmentation while guanethidine caused nerve fibre retraction. The uptake of these drugs by the nerve cell bodies and their processes is discussed in relation to their toxic effects.

Tissue culture
Sympathetic neurons
Axon retraction
Axon damage
Guanethidine
6-Hydroxydopamine

Introduction

In newborn animals, both 6-hydroxydopamine ngeletti and Levi-Montalcini, 1970a; Angeletti, 971; Eränkö and Eränkö, 1971b, 1972) and guan-hidine (Eränkö and Eränkö, 1971a,c) cause a chem-al sympathectomy. The effect on the adult sym-thetic nervous system, however, reveals some basic fferences in the mode and site of action of these two drugs. 6-Hydroxydopamine (6-OHDA) causes extensive depletion of catecholamines and damage to terminal nerve fibres without affecting the cell bodies so that after several weeks new nerve processes are formed (Thoenen and Tranzer, 1968; Tranzer and Thoenen, 1968). Guanethidine produces a chemical sympathectomy where less than 2% of the nerve cell bodies remain in the superior cervical ganglion following a 6 week chronic treatment, a situation which persists for at least four months following the cessation of treatment (Burnstock et al., 1971). Furthermore, the adrenergic nerve supply to the male reproductive tract is particularly resistant to 6-OHDA, even in newborn animals (Thoenen and Tranzer, 1968; Angeletti and Levi-Montalcini, 1971), but is particularly susceptible to guanethidine (Gannon et al., 1971; Evans et al., 1972). Thus, it was of interest to investigate further the mechanisms by which these two drugs produced a similar end result in newborn animals. Using tissue culture it is possible to determine whether these drugs act directly on adrenergic neurons or indirectly via other mechnisms in the body. In addition, it is possible to continuously moni-

This study was supported by the National Health and Medical Research Council, the National Heart Foundation of Australia and the Australian Research Grants Committee.

Holder of a Commonwealth of Australia Postgraduate ward.

University of Melbourne Senior Research Fellow, Septem-er 1971–August 1972. Present address: Department of natomy, University of Helsinki, Siltavuorenpenger, Hel-nki, Finland 00170.

Sunshine Foundation and Rowden White Overseas Research ellow in the University of Melbourne, September 1971–ugust 1972. Present address: Department of Anatomy, Uni-rsity of Helsinki, Siltavuorenpenger, Helsinki, Finland 0170.

ır the sequence of events which occur during degen-
ation. Previous studies in culture indicated the
rtolytic effect of 6-OHDA on human and mouse
euroblastoma cells (Angeletti and Levi-Montalcini,
970b) and the lack of toxic effect of doses of guan-
hidine up to 36 mg/l on newborn rat sympathetic
eurons (Eränkö et al., 1972). Therefore, this study
as undertaken in order to compare the actions of a
ide range of concentrations of 6-OHDA and guan-
hidine on cultures of newborn rat and embryo
ick sympathetic ganglia.

Materials and methods

1. Culture technique

Paravertebral sympathetic chain ganglia from 0 to
day old Sprague–Dawley rats and 17 day old
ıstolope–White Leghorn cross chick embryos were
ltured in modified Rose chambers by the method
scribed previously (Chamley et al., 1972). Trypsin
.125%) was often used to facilitate the removal of
e connective tissue sheath surrounding the ganglia.
fore addition of the two drugs, the cultures were
ubated at 37°C for 5–7 days in Medium 199, 80%
alk et al., 1954) and foetal calf serum, 20%, supple-
ented with 0.05 units/ml insulin, 100 units/ml
nicillin G, 5 mg/ml glucose and 1 unit/ml nerve
owth factor (Burroughs–Wellcome, England).

2. Preparation and administration of 6-hydroxydopamine

6-Hydroxydopamine hydrochloride (6-OHDA,
otec, Sweden) was added to both the rat and chick
nglion cultures at concentrations of 20, 40 and
mg/l (0.097, 0.195, 0.389 mM/l respectively) and
100 mg/l (0.486 mM/l) for the chick only. Oxida-
n of 6-OHDA was prevented by the addition of
orbic acid (Angeletti and Levi-Montalcini, 1970b),
stronger reducing agent. It was originally used at
0 mg/l, but as this proved to be toxic (see Results),
concentration was reduced to either 20 or
mg/l. All solutions were prepared by weighing
t the 6-OHDA and ascorbic acid and dissolving
m together in Hanks balanced salt solution (HBSS,
nks and Wallace, 1949). The combined solution was
rilized by 0.2 μ Millipore filtration, dilured with
culture medium and injected immediately into
the chambers. The cultures were then returned to the incubator for $1\frac{1}{2}$ hr or overnight (20 hr) before replacing the 6-OHDA with the control medium. As the drugs were dissolved in cold medium, approximately $\frac{1}{2}$ hr elapsed before the cultures reached the incubation temperature. Two series of control cultures were maintained. The first was incubated with the normal growth medium and the second with ascorbic acid at the same concentration as was present in the 6-OHDA–ascorbic acid test solution. The medium was changed in both sets of controls at the same time as the test cultures and the ascorbic acid in the second group replaced by control medium at times corresponding to those for the 6-OHDA containing cultures.

Observations were made with phase contrast optics, using a Zeiss Standard RA microscope. Several fields were selected to show explant neurons, migratory neurons, nerve fibres and glial and connective tissue cells. These fields were marked and photographs (Zeiss Ikon camera) taken before adding the drug and then at various times, from half an hour after its addition through to 9 days after its replacement with normal growth medium.

2.3. Preparation and administration of guanethidine

Rat cultures only were treated with 10, 50 and 100 mg/l guanethidine sulphate (CIBA) (0.040, 0.202, 0.404 mM/l respectively) which was dissolved in HBSS, sterilized by filtration and diluted in the culture medium. The condition of the neurons, nerve fibres and associated cells was monitored before addition of the drug and after $1\frac{1}{2}$ hr, 1 day and 2 days exposure to it, the medium being renewed every 24 hr to ensure its continuous availability. Control cultures contained the normal medium described above. In one experiment, using 100 mg/l guanethidine, the drug was replaced by the control medium after 1, 2 and 3 days, in order to test the capacity for recovery after these increasing exposure times.

2.4. Assessment of effects of drugs

Cells showing rounded cell bodies, vacuolated cytoplasm and the withdrawal or fragmentation of processes were considered damaged, while those with picnotic nuclei and ruptured cell membranes were regarded as dead. Changes in the cultures were considered in four categories according to locality; (i)

nerve terminal growth cones, (ii) peripheral nerve fibre networks, (iii) proximal nerve fibre networks with associated migratory neurons, glial and connective tissue cells and (iv) nerve cell bodies which remained in the explants. Thus, differences in sensitivity of these areas to the drugs could be observed.

Results

3.1. Control

The normal appearance of the rat and chick sympathetic ganglion cultures was similar to that previously described (Chamley et al., 1972). N fibres, migratory neurons and associated glial connective tissue elements grew out from th plants and by 5–7 days these had formed dens works. Medium changes did not affect the appea of the cells or abolish the nerve fibre growth c Both the fibres and cells near the explant (prox and in the peripheral nerve networks showed co erable movement, even in the first hour after a um change.

The explants gradually flattened during the f days so that the neurons were readily visible thr out the experimental period. Both the nerve

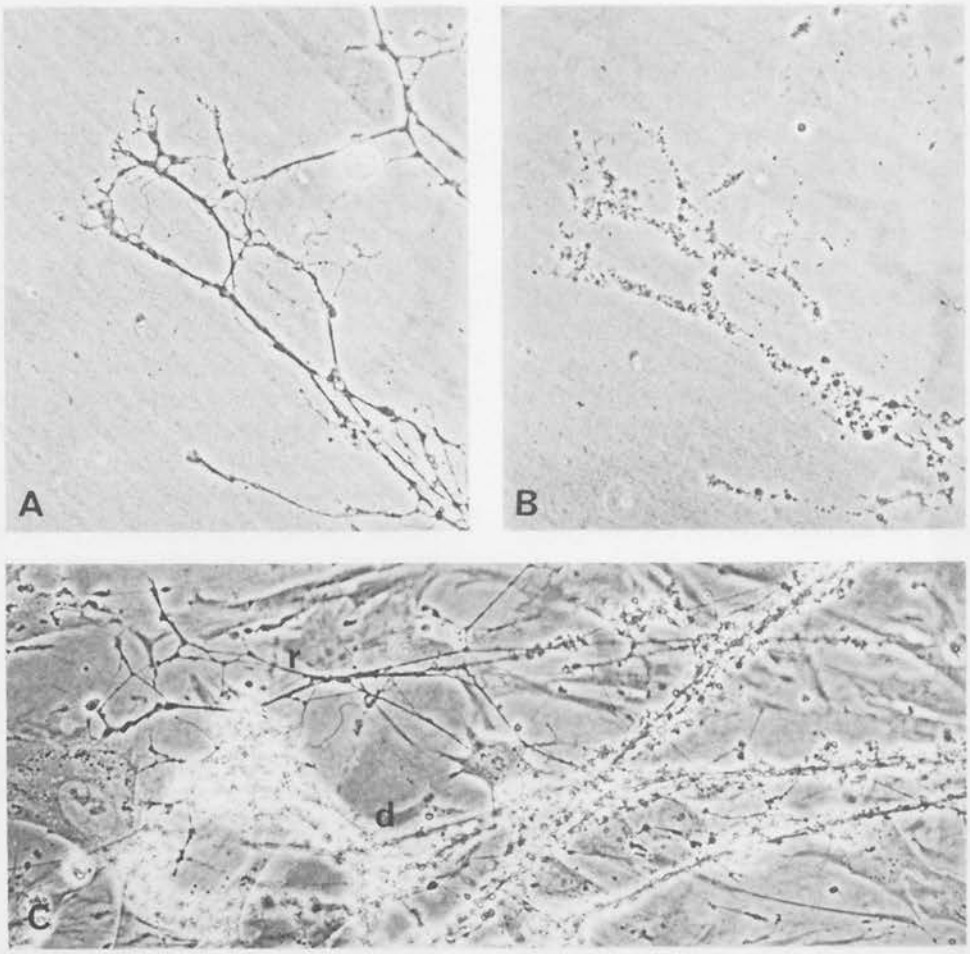


Fig. 1. Phase contrast photomicrographs illustrating 6-OHDA-induced fragmentation of nerve fibres and subsequent regene in chick embryo sympathetic ganglion cultures. A, 3-hr exposure to 20 mg/l 6-OHDA. Note that growth cones and fibres a intact and healthy. B, same field as A, after a further 17-hr exposure to 20 mg/l 6-OHDA. The fibres have fragmented in 'ghost' fibre of the resulting debris remains. C, 24 hr after a 20-hr exposure to 20 mg/l 6-OHDA. Healthy, regenerated fib can be seen beside 'blebby' and degenerated fibres (d). Magnification × 420.

dies which remained in or near the explant and se which migrated into the outgrowth appeared althy.

Ascorbic acid

Ascorbic acid at a concentration of 200 mg/l for r, after 4–6 days of control growth, had no apent effect on the chick or rat nerve cell bodies but used damage to growth cones in the chick and fragntation of fibres in the rat cultures. Furthermore, exposure for 20 hr resulted in fragmentation of fibres, damage and death of migratory neurons and damage to nerve cell bodies in the explants of both rat and chick cultures, although fibre regeneration occurred after the ascorbic acid was replaced with control medium.

Lower concentrations of ascorbic acid (20 and 40 mg/l) had no such deleterious effects and therefore these concentrations were selected for subsequent experiments with 6-OHDA.

Table 1
The effect of 6-hydroxydopamine on chick embryo cultures.

se g/l)	Length of exposure (hr)	Observation time *	Growth cones	Peripheral fibres	Proximal fibres	Nerve cells	
						In outgrowth	In explant
	1½	3 hr	+ [a]	–	–	–	–
		2 dy [d]	++	+B [b]	–	–	–
		7 dy	+	+B regeneration		–	–
	20	4 hr	++	+BF [c]	–	+	–
		20 hr	++	++BF	+B	++	–
		2 dy	+	+BF regeneration		++	–
		7 dy	–	Regeneration		++	+
	1½	2 hr	++	–	–	–	–
		20 hr	+	+B	–	–	–
	1½	½ hr	++	+B?	–	–	–
		1 hr	++	+BF	–	–	–
		3 hr	++	++BF	–	+	–
		20 hr	+	+BF regeneration		–	–
		2 dy	+	+B regeneration		–	–
		7 dy	+	+B regeneration		–	–
	20	4 hr	++	+BF	+B	+	–
		20 hr	++	++BF	+BF	+++	+
		2 dy	+	++BF regeneration	+B	++	++
		7 dy	+	+B regeneration	+B	+	++
)	1½	½ hr	++	+B	–	–	–
		2 dy	+	Regeneration		–	+
		7 dy	–	Regeneration		–	++
	20	4 hr	++	+BF		+	+
		20 hr	++	+++BF	++BF	+++	++
		2 dy	++	+++BF	++BF	All dead	++
ene s a		7 dy	–	Regeneration	+B	All dead	+++

ed ime elapsed since drug originally added to cultures.
fit, ++, +++: degree of damage.
, 'blebbing'.
, fragmentation.
y, day.

6-Hydroxydopamine

. Chick

here was a loss of growth cones at all concentra- studied.

itial drug-induced damage to peripheral fibres the form of "blebbing", i.e. minute ballooning e fibre membrane (fig. 1C). This was followed by nentation of the fibres so that the original posi- of the fibre became occupied by a 'ghost fibre' isting of the debris left after fragmentation 1A,B). Thus, where fibre regeneration had oc- d it was possible to see both degenerated and nerated fibres occupying the same area (fig. 1C). mentation resulted from longer exposure to 6- DA than that which caused "blebbing" (table 1). Higher concentrations and longer exposures pro earlier and progressively increasing effects (tab Regeneration of peripheral fibres occurred to va extents during the recovery in control medium all concentrations and exposures.

The proximal fibre network was more res than the peripheral network. Only at higher co trations or longer exposures to the drug did co rable damage occur. Amongst the cells asso with the proximal network, the migratory ne were the most susceptible to 6-OHDA, the (20 hr) exposures at 20 and 80 mg/l having a effect on some and, at 100 mg/l, on all of them. glial cells showed vacuolation and rounding their nuclear regions following the 20-hr periods and 100 mg/l although they recovered in n

Table 2
The effect of 6-hydroxydopamine on rat ganglion cultures.

)	Length of exposure (hr)	Observation time *	Growth cones	Peripheral fibres	Proximal fibres	Nerve cells	
						In outgrowth	In expl
	1½	2 hr	+?	–	–	–	–
		6 hr	+?	–	–	–	–
		1 dy [d]	+?	–	–	–	–
		4 dy	–	–	–	–	–
	20	2 hr	+?	–	–	–	–
		6 hr	+	+B [b]	+B	+?	–
		1 dy	++	++F [c]	++F	+	+
		4 dy	+	Regeneration		+	?
	1½	2 hr	+	+B	+B	+	–
		6 hr	+	++BF	++BF	++	+
		1 dy	++	+++F	+++F	+++	++
		4 dy	+	Regeneration		Dead	++
	1½	1 hr	+	+B	+B	–	+
		6 hr	++	++BF	++BF	+	++
		1 dy	Outgrowth collapsed close to explant				+++
		10 dy	+	Regeneration		++	+++
	20	1 hr	+	+B	+B		+
		6 hr	++	++F	++F	+++	+++
		1 dy	Outgrowth collapsed close to explant				
		10 dy	No regeneration		All cells dead		

ne elapsed since drug originally added to cultures.
++, +++: degree of damage.
'blebbing'.
fragmentation.
day.

medium. Fibroblasts, however, were unaffect 00 mg/l for 20 hr they ceased to move about freely nd time-lapse cinematography showed that their nor- ally active membranes had become immobilised. his effect was reversed on return to drug-free me- ium.

The nerve cell bodies in the explant were generally e last to be affected and damage occurred only af- r the long exposures to the drug. At 80 mg/l and 00 mg/l there appeared to be a decrease in the num- er of neurons present.

3.2. Rat

The differences in sensitivity of the four areas of e rat ganglion cultures to 6-OHDA were similar to ose of the chick cultures (table 2). Exposure to mg/l for $1\frac{1}{2}$ hr had little effect and the same con- ntration overnight mainly affected nerve fibres. owever, exposure to 40 mg/l for $1\frac{1}{2}$ hr caused exten- e damage with fragmentation and loss of fibres in e outgrowth and death of peripherally situated ex- ant neurons (fig. 2). Next day there was further fibre destruction and cell death in the explants, and after 4 days, although there was regeneration of new nerve fibres, the nerve network was sparse and the size of the explants decreased by about 50%.

A higher concentration of 80 mg/l, for $1\frac{1}{2}$ hr, caused extensive damage to both peripheral and proximal fibres and, $4\frac{1}{2}$ hr later, the fibrous and cellular outgrowth had collapsed in towards the explant, probably as a result of the removal of peripheral attachments through the loss of terminal fibres and growth cones. Glial cells and explant nerve cell bodies were also severely damaged. By 10 days, a few nerve fibres had regenerated although the majority of the neurons had been killed. A 20-hr exposure to 80 mg/l caused death of all cells in the culture. No regeneration was seen even after 9 days in the control medium.

3.4. Guanethidine

Rat ganglion cultures exposed to guanethidine sulphate showed a dose response in which sensitivity increased with distance from the explants (table 3).

At 10 mg/l the only observable effect, even after 2

Table 3
The effect of guanethidine on rat ganglion cultures.

se g/l)	Length of exposure (hr)	Observation time *	Growth cones	Peripheral fibres	Proximal fibres	Nerve cells	
						In outgrowth	In explant
	1½	1½ hr	–	–	–	–	–
	24	1 dy [d]	Growth rate less		–	–	–
	48	2 dy	Growth rate less		–	–	–
	1½	1½ hr	++R [c]	–	–	–	–
	24	1 dy	++R	+BR	+B [b]	+	–
	48	2 dy	++R	++BR	++BR	++	+
0	1½	1½ hr	++R	+R	–	–	–
	24	1 dy	++R	++BR	+BR	+	+
		2 dy	Regeneration		–	–	+
	48	2 dy	++R	+++BR	+++BR	++	++
		3 dy	Regeneration				++
	72	3 dy	++R	+++BR	+++BR	+++	+++
		4 dy	Some regeneration				+++

ime elapsed since drug originally added to cultures.
, ++, +++: degree of damage.
l, 'blebbing'.
l, retraction.
y, day.

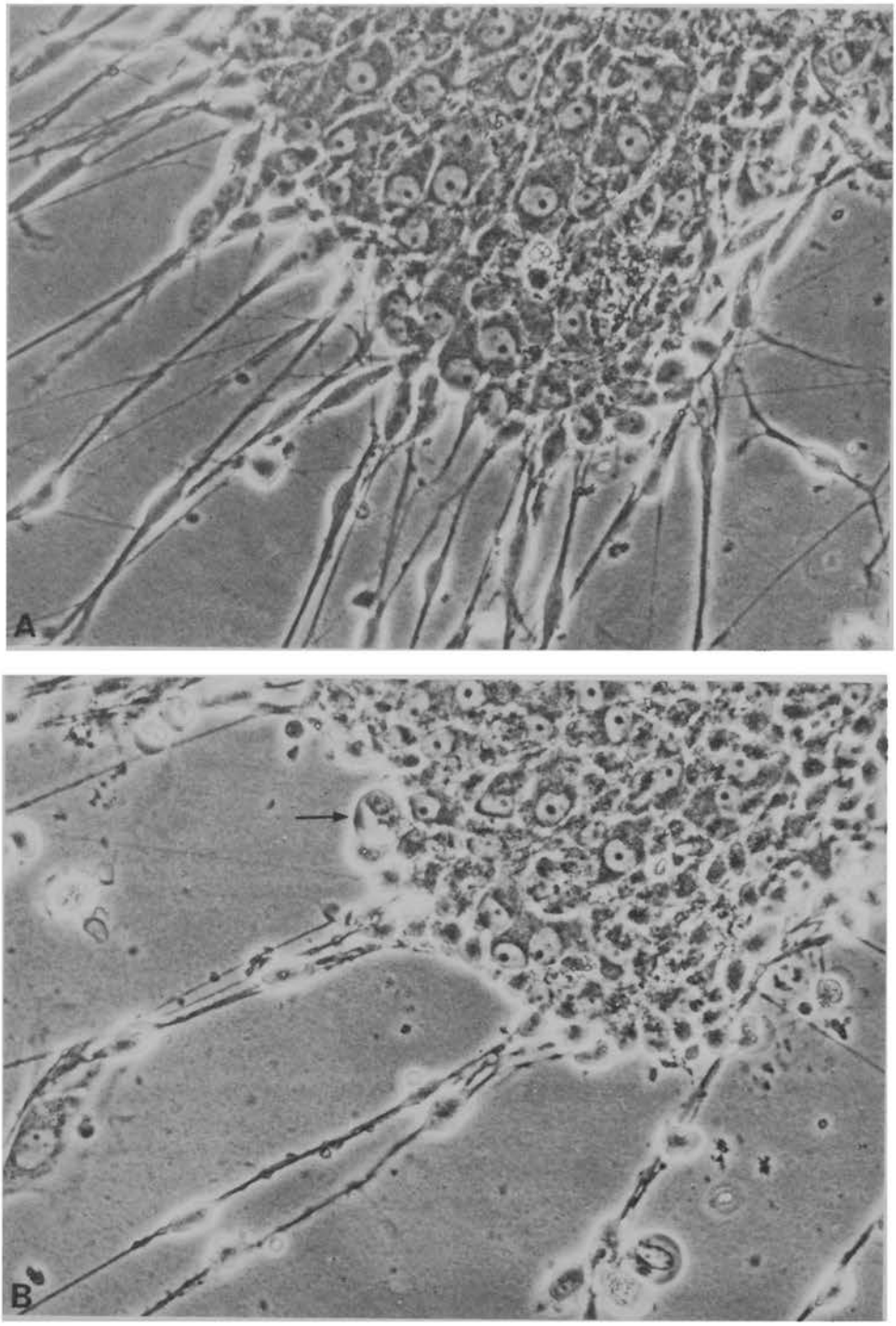
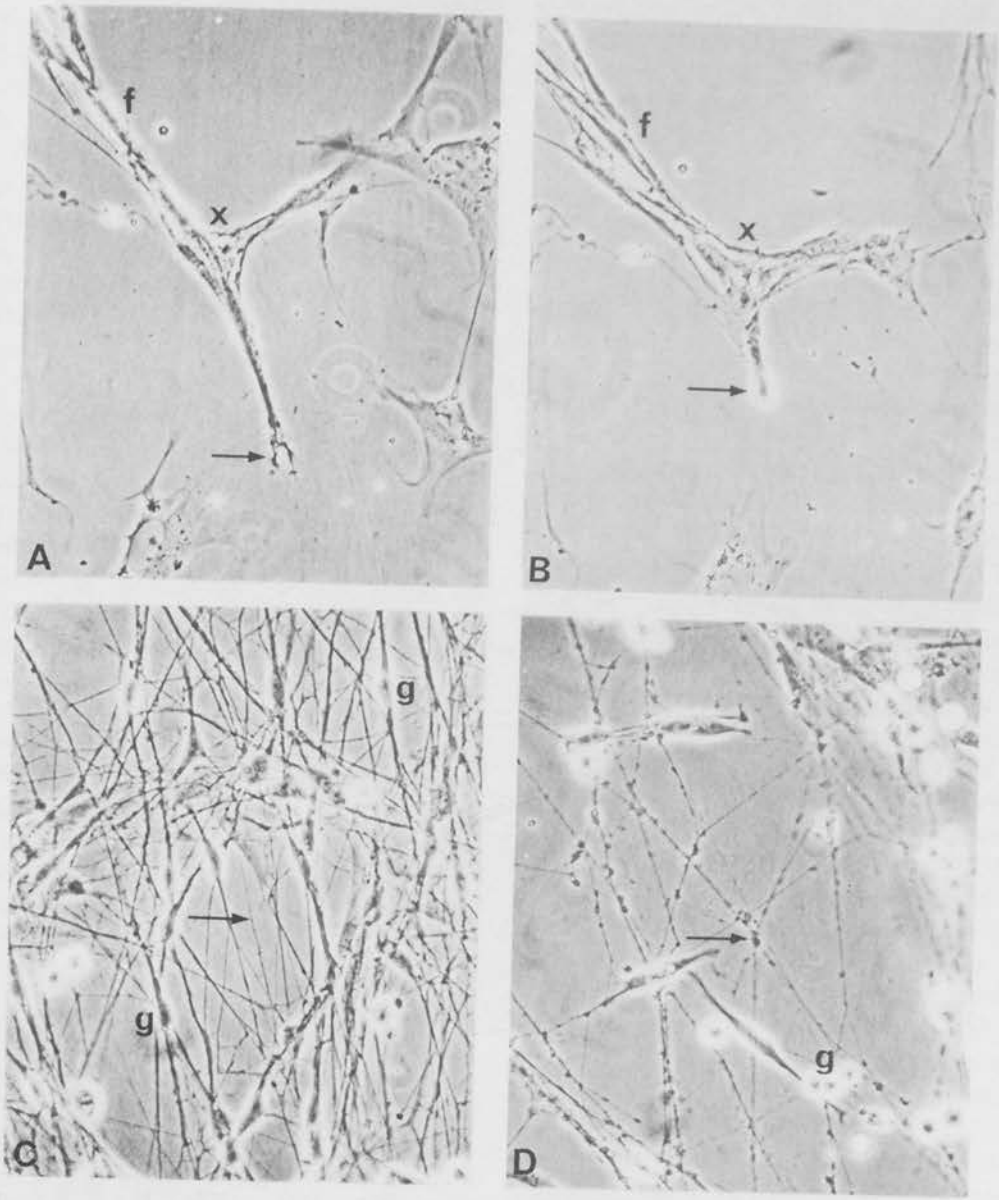


Fig. 2. Phase contrast photomicrographs of the same area of a newborn rat sympathetic ganglion culture before and after add of 6-OHDA into the culture medium. A, normal appearance of nerve cell bodies in the explant peninsula and numerous fibres emerging from it. B, after $1\frac{1}{2}$ hr exposure to 40 mg/l of 6-OHDA and a subsequent $4\frac{1}{2}$ hr in the control medium. No loss of many nerve fibres and the changes in the appearance of the peripheral nerve cells in the explant. One nerve cell body ballooning of the cell membrane is indicated by an arrow. Magnification × 400.



3. Phase contrast photomicrographs of nerve fibre retraction produced by guanethidine sulphate in newborn rat sympathetic glion cultures. A, terminal nerve fibres and growth cones before addition of guanethidine. The nerve fibre bundle (f) bifurcates X, one branch terminating in growth cones (arrow). B, same field as A, after $1\frac{1}{2}$ hr exposure to 100 mg/l guanethidine sulphate. growth cones and terminal fibres have retracted back towards the point where the bundle bifurcates. The halo around these racted fibres (arrow) is caused by the thickness of the tissue. C, fibrous and cellular outgrowth before addition of guanethidine. te nerve fibre network (arrow) and glial cells (g). D, same field as C after 2 days continuous exposure to 100 mg/l guanethidine. te that the number of nerve fibres present is greatly reduced and although they are 'blebby' (arrow), they are all intact and no gmented fibres can be seen. The round glowing objects (g) are dead or damaged glial and connective tissue cells. Magnification ad 60.

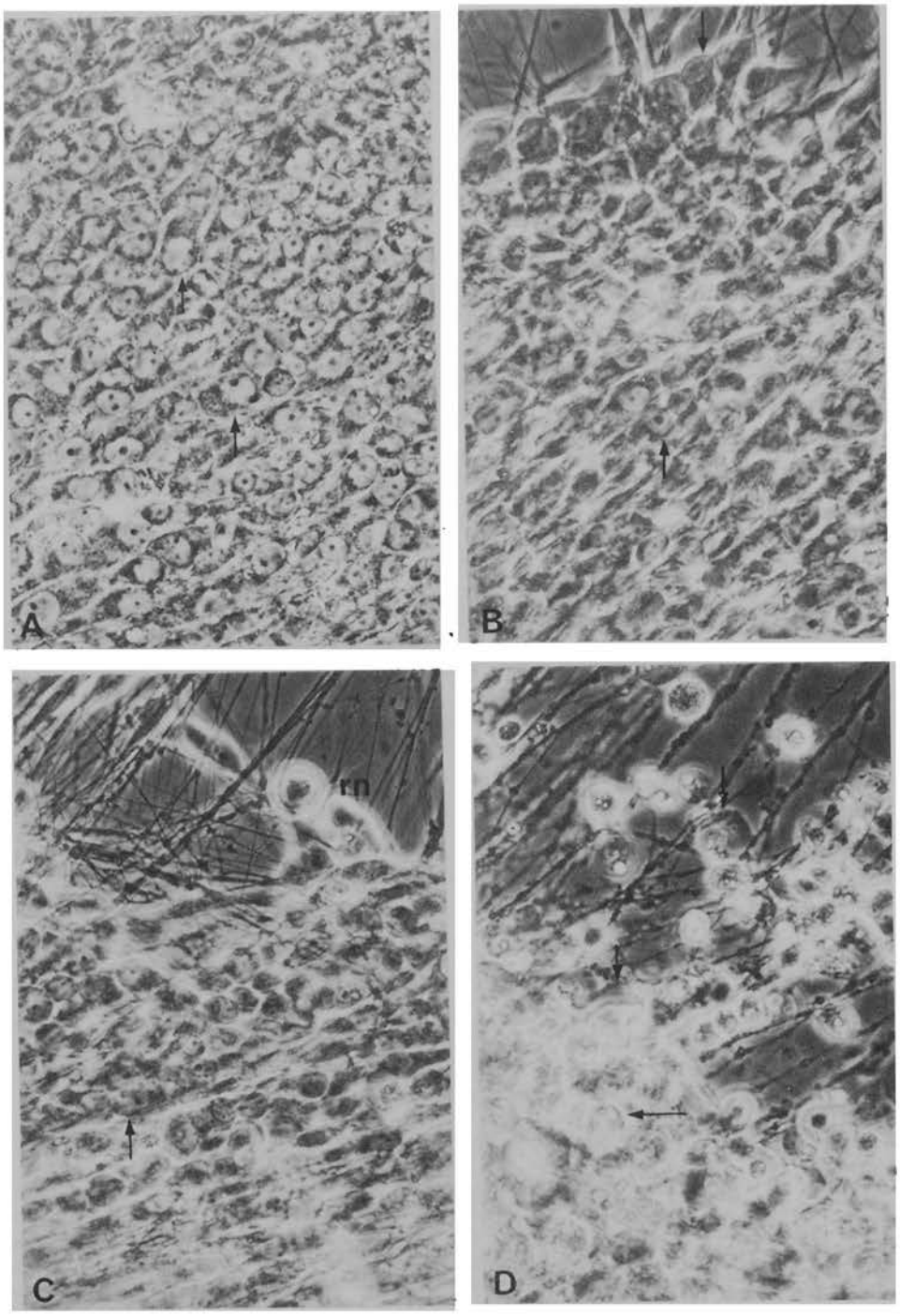


Fig. 4. A series of phase contrast photomicrographs of the same field showing guanethidine-induced damage to newborn rat sympathetic nerve cell bodies. A, before addition of guanethidine, healthy nerve cell bodies (arrows) are clearly visible. B, after 1 day exposure to 100 mg/l guanethidine, nerve cell bodies still look healthy (arrows). C, after 1 day exposure to 100 mg/l guanethidine, healthy neurons are still present (arrow) although many of the cells are rounding up (rn). D, after 2 days exposure, the majority of the peripherally situated explant neurons are dead. Note the extensive ballooning of cell membranes (arrows). Magnification x

days continuous exposure, was a decrease by about half in the rate of growth of the peripheral fibres. Both 50 and 100 mg/l guanethidine caused loss of growth cones and retraction of many terminal fibres after only $1\frac{1}{2}$ hr exposure (fig. 3A,B).

Fibre damage was characterised by 'blebbing' and retraction (fig. 3). The latter was gauged by sequential monitoring of marked fields originally containing peripheral fibres and their terminations. The fibres were retracted back towards the explants leaving the marked fields empty. Furthermore, there was retraction of more than 50% of the original number of fibres in the outgrowth after 2 days exposure to 100 mg/l of guanethidine sulphate without any signs of the debris resulting from fibre fragmentation which was a common feature of 6-OHDA cultures (fig. 3C,D). However, reappearance of growth cones and regrowth of fibres was evident 24 hr after 1 and 2 day exposures to 100 mg/l and even after a 3 day exposure the remaining fibres showed recovery.

Glial cells and migratory neurons showed some vacuolation and rounding up of their nuclear regions after 50 mg/l guanethidine for 1 and 2 days, while explant neurons showed only slight rounding of their cell bodies. Exposure to 100 mg/l for 1 and 2 days caused increased damage and death of glial cells and migratory neurons and, after 2 days, of the peripherally situated explant neurons (fig. 4). A longer exposure (3 days) at 100 mg/l resulted in further cell death in the explants although some neurons were still visible in the central areas.

Normal looking fibroblasts could be found after all doses and exposures, although after 50 and 100 mg/l some had become vacuolated and they appeared to move about more slowly than the fibroblasts in the control cultures.

Discussion

Guanethidine and 6-hydroxydopamine had a direct effect on both newborn rat and embryo chick sympathetic ganglion cultures. The extent of cellular damage depended on the concentration of the drug tested and the duration of its application. Four areas of the cultures: (i) nerve terminal growth cones, (ii) peripheral nerve fibre networks, (iii) proximal nerve fibre networks with associated migratory neurons and glial cells, and (iv) explant nerve cell bodies, showed differing susceptibilities to both 6-OHDA and guanethidine; sensitivity increasing with distance from the explant.

A major difference between the effect produced by 6-OHDA and guanethidine was the type of damage to the fibres. Although ballooning of the axonal membrane occurred in both cases, 6-OHDA subsequently caused fragmentation while guanethidine produced retraction of nerve fibres. In the rat cultures containing 6-OHDA, the observed collapse of the outgrowth towards the explant was probably passive and a result of the loss of peripheral attachments through damage to fibre terminals, as both fibres and cells were involved. Fibre retraction after guanethidine, however, occurred without affecting the positions of the fibroblasts and glial cells, perhaps indicating the presence of contractile elements in the fibres.

Regeneration of fibres following the cessation of treatment with 6-OHDA appeared to differ from that occurring after guanethidine. In the former case, it seems likely that regenerating fibres were produced by collateral sprouting from the original fibres, proximal to the damaged regions, as both degenerated and regenerating fibres could be seen together. Recovery from the degenerative effects of 6-OHDA in vivo also appears to depend on regenerative axon sprouting (Thoenen et al., 1970). In contrast, in guanethidine-treated cultures, regeneration probably occurred by recovery and extension of the previously retracted fibres, since no damaged fibres were ever observed.

The neuronal population of the cultured ganglia showed a heterogeneous response to both drugs. The neuronal cell bodies which migrated into the outgrowth were generally the first and most severely affected. This could result from their exposure to the drugs being greater than that of the cells remaining clumped together in the explant. In addition, Chamley et al. (1972) proposed that these migratory neurons represent a more immature element of the ganglion. Therefore, their greater sensitivity would also be consistent with an undifferentiated state. The survival of a small percentage of the neuronal population following 20 hr of 100 mg/l 6-OHDA in the chick, 1 hr of 80 mg/l 6-OHDA in the rat and 3 days of 100 mg/l guanethidine in the rat suggests the

presence of a small number of highly resistant cells. Similar cell survival after chronic treatments in vivo with both drugs has been reported (Burnstock et al., 1971; Eränkö and Eränkö, 1971a; Eränkö and Eränkö, 1972). Angeletti (1971) and Eränkö and Eränkö (1972) attributed this survival after 6-OHDA to an advanced state of differentiation since adult neurons are resistant to the effects of this drug (Tranzer and Thoenen, 1968). However, following chronic guanethidine treatment, ultrastructural studies revealed that all neurons in the superior cervical ganglion showed signs of damage and that cells in all stages of degeneration were present at any one time (Heath et al., 1972). Therefore, the survival of some cells following guanethidine treatment in culture may represent variability among neurons in the rate at which they are damaged.

Eränkö et al. (1972) reported neuronal outgrowth in the presence of 36 mg/l of guanethidine throughout the culture period. However, in the present study, inhibition of growth cones was observed when 10 mg/l of guanethidine was added to a culture which had been allowed to form a nerve fibre net in the absence of any drug. When guanethidine is present throughout the culture period nerve fibres are regenerated only by neurons resistant to this drug. In contrast, the nerve fibre net formed in cultures without the drug originates to a great extent from neurons sensitive to it and is therefore easily damaged by subsequent addition of the drug.

Cultures rat sympathetic neurons were more sensitive to 6-OHDA than chick neurons. If amine uptake and storage mechanisms were more efficient in mammals than in birds then an increased susceptibility to drugs, like 6-OHDA, which selectively act on adrenergic neurons would be expected. Also, the developmental stage of the chicken neurons (17 day embryo) may be more advanced than that of the newborn rat, resulting in greater resistance to 6-OHDA.

6-Hydroxydopamine is taken up into the nerve terminals of adult animals, against a concentration gradient, by the active amine membrane pump (Malmfors and Sachs, 1968; Jonsson and Sachs, 1970; Jonsson, 1971). Degeneration occurs only when a critical concentration is reached (Bennett et al., 1970; Furness et al., 1970; Jonsson and Sachs, 1970). There is indirect evidence that 6-OHDA is also taken up by nerve cell bodies in newborn animals, Angeletti and Levi-Montalcini (1970b) having reported that OHDA prevented the uptake of labelled dopam and norepinephrine by mouse and human neurob toma cell bodies and their processes. The lack of fect of 6-OHDA on adult nerve cell bodies may in cate, therefore, that with subsequent differentiat the activity of the amine pump may be reduced the interior of the nerve cell body may be less s ceptible to 6-OHDA.

In this study the fibre terminals were more susc tible to damage by 6-OHDA than the cell bod England and Goldstein (1969) demonstrated, by m suring the degree of uptake of labelled amines, t the amine membrane pump in chick embryo sy pathetic neurons was more active in the axons tha the cell bodies. Thus, if 6-OHDA uptake occur both the cell bodies and the nerve terminals, then terminals would be first to accumulate damaging c centrations of the drug.

Guanethidine may also be accumulated by the minal fibres and the cell bodies of adrenergic neur Its effect on isolated fibres has been stu (Schanker and Morrison, 1965; Obianwu et al., 19 Mitchell and Oates, 1970; Gulati and Jaykar, 19 Juul and Sand (1971) reported its accumulation in superior cervical ganglion and Jensen-Holm and J (1970a,b) found degenerative effects of guanethi on axotomized neurons. We found susceptibilit guanethidine at the light microscope level to be gre in the fibre terminals than in the nerve cell bodie for 6-OHDA. This may be due to greater uptak amines in the terminals than in the cell bodies (I land and Goldstein, 1969). In addition, howe guanethidine inhibits oxidative phosphorylation isolated mitochondria (Malmquist and Oates, 19 and causes structural damage to mitochondria adrenergic neurons in the rat (Jensen-Holm and J 1971; Heath et al., 1972). Hence, the greater susce bility of nerve terminals to guanethidine may als due to the more rapid elimination of energy sour as presumably fewer mitochondria occur in the n terminals than in the cell bodies. Alternatively, d age in the cell bodies may prevent the axonal fl from the cell bodies to the terminals, of mate essential for maintenance and repair.

Acknowledgement

The authors wish to thank Miss Janet McConnell for skillful technical assistance and Mr. K.G. Hill for reading and criticizing the manuscript.

References

Angeletti, P.U., 1971, Chemical sympathectomy in newborn animals, Neuropharmacol. 10, 55.

Angeletti, P.U. and R. Levi-Montalcini, 1970a, Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine, Proc. Nat. Acad. Sci. U.S. 65, 114.

Angeletti, P.U. and R. Levi-Montalcini, 1970b, Cytolytic effect of 6-hydroxydopamine on neuroblastoma cells, Cancer Res. 30, 2863.

Angeletti P.U. and R. Levi-Montalcini, 1971, Growth regulation of the sympathetic nervous system: immunosympathectomy and chemical sympathectomy, European J. Clinical Biol. Res. 16, 866.

Bennett, T., G. Burnstock, J.L.S. Cobb and T. Malmfors, 1970, An ultrastructural and histochemical study of the short-term effects of 6-hydroxydopamine on adrenergic nerves in the domestic fowl, Brit. J. Pharmacol. 38, 802.

Burnstock, G., B.K. Evans, B.J. Gannon, J.W. Heath and V.B. James, 1971, A new method of destroying adrenergic nerves in adult animals using guanethidine, Brit. J. Pharmacol. 43, 295.

Chamley, J.H., G.E. Mark, G.R. Campbell and G. Burnstock, 1972, Sympathetic ganglia in culture. I. Neurons, Z. Zellforsch. 135, 315.

England, J.M. and M.N. Goldstein, 1969, The uptake and localization of catecholamines in chick embryo sympathetic neurons in tissue culture, J. Cell Sci. 4, 677.

Eränkö, L. and O. Eränkö, 1971a, Effect of guanethidine on nerve cells and small intensely fluorescent cells in sympathetic ganglia of newborn and adult rats, Acta Pharmacol. Toxicol. 30, 403.

Eränkö, O. and L. Eränkö, 1971b, Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of the pineal body of the rat after chemical sympathectomy with 6-hydroxydopamine, Histochem. J. 3, 357.

Eränkö, O. and L. Eränkö, 1971c, Histochemical evidence of chemical sympathectomy by guanethidine in newborn rats, Histochem. J. 3, 451.

Eränkö, L. and O. Eränkö, 1972, Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat, Acta Physiol. Scand. 84, 115.

Eränkö, L., C.E. Hill, O. Eränkö and G. Burnstock, 1972, Lack of toxic effect of guanethidine on nerve cells and small intensely fluorescent cells in cultures of sympathetic ganglia of newborn rats, Brain Res. 43, 501.

Evans, B.K., B.J. Gannon, J.W. Heath and G. Burnstock, 1972, Long lasting damage to the internal male genital organs and their adrenergic innervation in rats following chronic treatment with the antihypertensive drug guanethidine, Fertil. Steril. 23, 657.

Furness, J.B., G.R. Campbell, S.M. Gillard, T. Malmfors, J.L.S. Cobb and G. Burnstock, 1970, Cellular studies of sympathetic denervation produced by 6-hydroxydopamine in the vas deferens, J. Pharmacol. Exptl. Ther. 174, 111.

Gannon, B.J., T. Iwayama, G. Burnstock, J. Gerkens and M.L. Mashford, 1971, Prolonged effects of chronic guanethidine treatment on the sympathetic innervation of the genitalia of male rats, Med. J. Aust. 2, 207.

Gulati, O.D. and S. Jaykar, 1971, Factors affecting the action of guanethidine on adrenergic neurones, Brit. J. Pharmacol. 42, 352.

Hanks, J.H. and R.E. Wallace, 1949, Relation of oxygen and temperature in the preservation of tissues by refrigeration, Proc. Soc. Exp. Biol. (N.Y.). 71, 196.

Heath, J.W., B.K. Evans, B.J. Gannon, G. Burnstock and V.B. James, 1972, Degeneration of adrenergic neurons following guanethidine treatment: an ultrastructural study, Virchows Arch. Abt. B Zellpath. 11, 182.

Jensen-Holm, J. and P. Juul, 1970a, The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and protein, Acta Pharmacol. Toxicol. 28, 270.

Jensen-Holm, J. and P. Juul, 1970b, The effects of guanethidine, pre- and postganglionic nerve division on the rat superior cervical ganglion: cholinesterases and catecholamines (histochemistry), and histology, Acta Pharmacol. Toxicol. 28, 283.

Jensen-Holm, J. and P. Juul, 1971, Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration, Acta Pharmacol. Toxicol. 30, 308.

Jonsson, G., 1971, Studies on the uptake and accumulation of ^{3}H-6-hydroxydopamine in adrenergic nerves, in: 6-Hydroxydopamine and Catecholamine Neurons, ed. by T. Malmfors and H. Thoenen, (North-Holland Publishing Company, Amsterdam) p. 243.

Jonsson, G. and C. Sachs, 1970, Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons, European J. Pharmacol. 9, 141.

Jonsson, G. and C. Sachs, 1972, Degenerative and nondegenerative effects of 6-hydroxypamine on adrenergic nerves, J. Pharmacol. Exptl. Therap. 180, 625.

Juul, P. and O. Sand, 1971, Guanethidine determination in rat sympathetic ganglia following prolonged administration, Acta Pharmacol. Toxicol. 29, Supp. 4, 25.

Malmfors, T. and C. Sachs, 1968, Degeneration of adrenergic nerves produced by 6-hydroxydopamine, European J. Pharmacol. 3, 89.

Malmquist, J. and J.A. Oates, 1968, Effects of adrenergic neuronblocking guanidine derivatives on mitochondrial metabolism, Biochem. Pharmacol. 17, 1845.

Mitchell, J.R. and J.A. Oates, 1970, Guanethidine and related

agents. I. Mechanism of the selective blockade of adrenergic neurons and its antagonism by drugs, J. Pharmacol. Exptl. Therap. 172, 100.

Obianwu, H.W., R. Stitzel and P. Lundborg, 1968, Subcellular distribution of (^{3}H)-amphetamine and (^{3}H)-guanethidine and their interaction with adrenergic neurons, J. Pharm. Pharmacol. 20, 585.

Pick, J., 1970, The Autonomic Nervous System. Morphological, Comparative, Clinical and Surgical Aspects (J.B. Lippincott Company, Philadelphia, Toronto).

Salk, E.S., J.S. Youngner and E.N. Ward, 1954, Use of color change of phenol red as the indicator in titrating poliomyelitis viris or its antibody in a tissue culture system. Appendix. Method of preparing medium 199, Amer. J. Hyg. 60, 214.

Schanker, L.S. and A.S. Morrison, 1965, Physiological disposition of guanethidine in the rat and its uptake by h slices, Intern. J. Neuropharmacol. 4, 27.

Thoenen, H. and J.P. Tranzer, 1968, Chemical sympat tomy by selective destruction of adrenergic nerve end with 6-hydroxydopamine, Naunyn-Schmiedebergs A Pharmakol. 261, 271.

Thoenen, H., J.P. Tranzer and G. Haeusler, 1970, Chem sympathectomy with 6-hydroxydopamine. in: New pects of Storage and Release Mechanisms of Catech mines, eds. H.J. Schümann and G. Kroneberg (Sprin Verlag, Berlin–Heidelberg–New York) p. 130.

Tranzer, J.P. and H. Thoenen, 1968, An electron microsc study of selective, acute degeneration of sympath nerve terminals after administration of 6-hydroxy amine, Experientia (Basel) 24, 155.

Histochemical Journal, 5 (1973), 479–485

Histochemically demonstrable increase in the catecholamine content of the carotid body in adult rats treated with methylprednisolone or hydrocortisone

O. KORKALA, O. ERÄNKÖ, S. PARTANEN, L. ERÄNKÖ *and* A. HERVONEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland.

Received 25 April 1973

Synopsis. Carotid bodies of adult albino rats were examined using the formaldehyde-induced fluorescence (FIF) method for the demonstration of fluorogenic monoamines and staining with 1% Toluidine Blue for morphological observations.

In the carotid body of normal controls, most glomus (principal or type I) cells exhibited a FIF presumably due to catecholamines. The intensity of the fluorescence was weak in most cells, while some glomus cells were non-fluorescent and others exhibited a moderate or intense FIF. The sustentacular (satellite, supporting or type II) cells were essentially non-fluorescent.

One week after the administration of a single intraperitoneal injection of the long-acting glucocorticoid 6-methylprednisolone sypionate (400 mg/kg) or after seven intraperitoneal injections of the water-soluble glucocorticoid hydrocortisone sodium succinate (40 mg/kg daily for a week), a distinct increase was observed in the FIF of the glomus cells. No non-fluorescent glomus cells were observed after treatment with either glucocorticoid, and the intensity of most fluorescent glomus cells was moderate or intense.

It is concluded that glucocorticoids cause an increased storage of catecholamines in the glomus cells of the carotid body of the adult rat, an observation of interest in view of the fact that such changes due to glucocorticoids have as yet been reported only in catecholamine-storing cells of newborn rats.

Introduction

Small, intensely fluorescent (SIF) cells were first observed in the superior cervical ganglion of the rat (Eränkö & Härkönen, 1963, 1965). These cells are now known to be a constant feature in the sympathetic ganglia of several species (Matthews & Raisman, 1969; Eränkö & Eränkö, 1971; Eränkö, 1972; Kanerva, 1972).

In newborn rats, hydrocortisone causes a pronounced increase in the number of SIF

cells in the sympathetic ganglia and in the Organ of Zuckerkandl (Eränkö & Eränkö, 1972). Glucocorticoids also prevent the degeneration of the Organ of Zuckerkandl (Lempinen, 1964) and the decrease in the intensity of the catecholamine fluorescence from the glomus cells of the carotid body (Hervonen *et al.*, 1972), which normally occur within two weeks after birth in the rat.

The present study was carried out to study the effect of glucocorticoids on the histochemically demonstrable catecholamines in the carotid body of adult rats.

Material and methods

Seventeen adult rats of the Sprague-Dawley strain, each weighing about 180–220 g, were used. Eleven of these rats were given a single intraperitoneal injection of 400 mg/kg body weight of 6-methylprednisolone sypionate in oil suspension (Urbason Depot, Manoject, Hoechst), while the remaining six rats were daily given intraperitoneal injections of 40 mg/kg hydrocortisone sodium succinate (Hydro-Adreson, Organon) for 7 days.

The rats were killed one week after the only methylprednisolone injection or the first hydrocortisone injection, respectively, together with twelve untreated controls. The whole carotid bifurcation area, including the superior cervical ganglion, was removed. The tissue blocks were frozen in propane cooled with liquid nitrogen and dried for 5–6 days at −40°C in vacuum with a phosphorus pentoxide trap close by the tissue holder.

For the histochemical demonstration of catecholamines, the formaldehyde-induced fluorescence method was used according to the general rules presented by Eränkö (1967). Exposure to formaldehyde vapour, generated from paraformaldehyde powder which had been equilibriated with air of 60% relative humidity, was carried out for 60 min at 60°C and thereafter for 30 min at 80°C. The specimens were then embedded in a mixture of Epon and Araldite resins (Eränkö & Eränkö, 1971) and cut at 5 μm with a Pyramitome (LKB).

For fluorescence microscopy, a Leitz Ortholux microscope equipped with the Ploem (1971) epi-illuminator was used. The following Leitz filter combination was employed: BG 38 (heat-absorbing filter), BG3 (blue filter), TAL 408 (interference filter) and K 470 (u.v. cut-off filter). As a light source an Osram HBO 200 mercury lamp was used.

Sections adjacent to those examined by fluorescence microscopy were stained with a 1% aqueous solution of Toluidine Blue and examined in transmitted light for general morphological features. The approximate proportion of non-fluorescent and weakly, moderately and intensely fluorescent glomus cells was visually assessed to the nearest 10% from four central sections of the carotid body of each animal.

Results

Normal controls

In serial sections, the carotid body was detected at the branching site of the common carotid artery. The stroma of the carotid body was surrounded by a loose capsule of connective tissue. The glomus cells (principal, type I or chief cells) of the carotid body formed coiled and anastomosing cell cords separated from each other by thick-walled, round capillaries and narrow spaces with sustentacular (type II or supporting) cell processes and connective tissue.

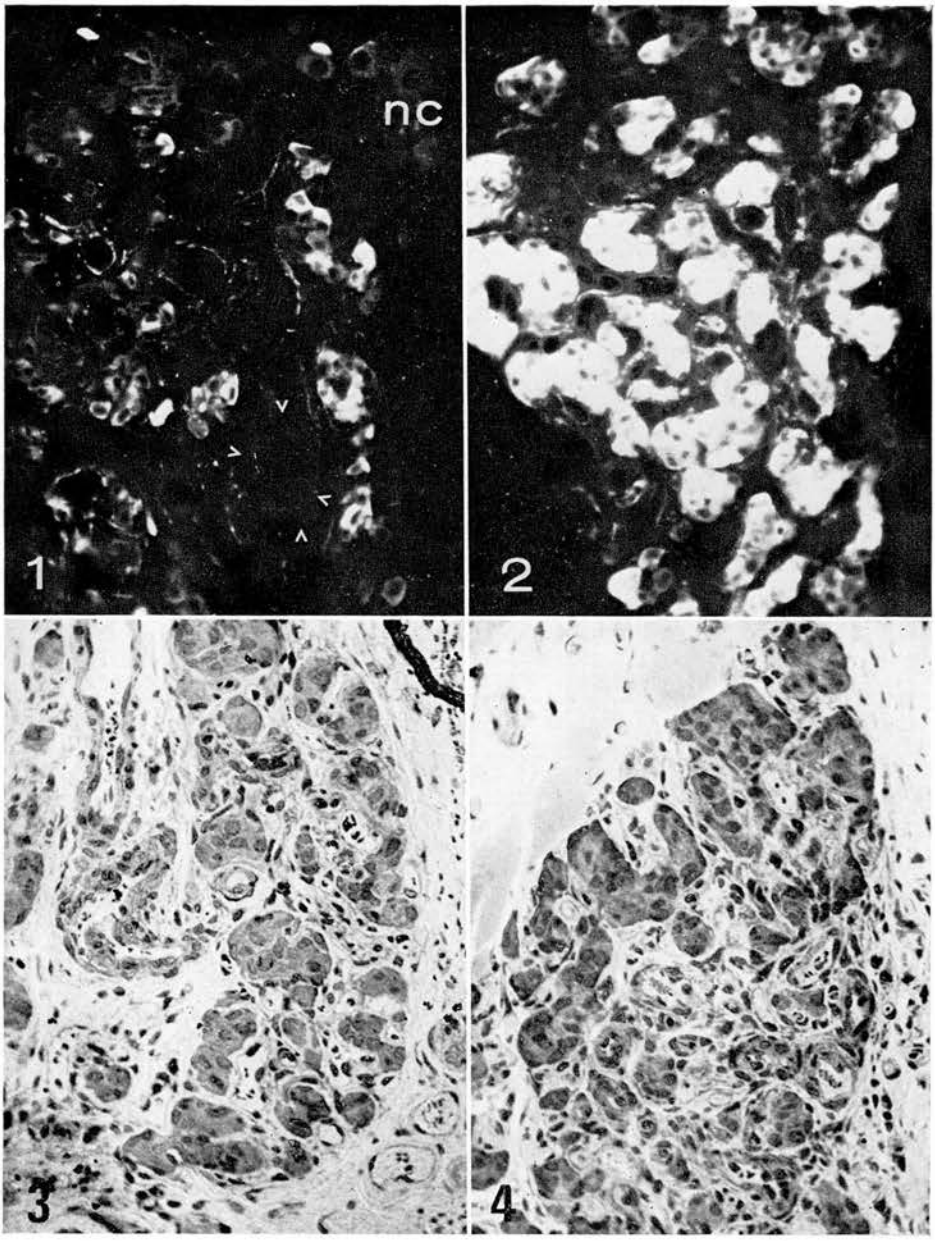


Figure 1. Formaldehyde-induced fluorescence in the carotid body of a normal adult rat. Most glomus cells exhibit a weak fluorescence, and only a few cells with an intense fluorescence are seen. A non-fluorescent chord of glomus cells is marked with 4 v's. Note the fluorescent nerve fibres in the vicinity of the glomus cells and a group of nerve cells (nc) near the carotid body. × 200

Figure 2. Formaldehyde-induced fluorescence in the carotid body of a methylprednisolone-treated adult rat. Note the dense chords of intensely fluorescent cells. None of the glomus cells is non-fluorescent. The figure has been taken and developed under conditions similar to those used for Fig. 1. × 200

Figure 3. A typical section of the carotid body stained with Toluidine Blue. The glomus cells show round, weakly stained nuclei and a weakly stained cytoplasm, while the sustentacular cells show irregular, dark nuclei. × 200

Figure 4. Corresponding view of the carotid body of a methylprednisolone-treated rat. No evidence for an increase in the size or number of the glomus cells can be seen. × 200

Fig. 1 illustrates the distribution of the formaldehyde-induced fluorescence (FIF) in a normal carotid body. The intensity of the FIF greatly varied from one glomus cell to another. They were divided into four groups according to the fluorescence intensity (Table 1). About 10% of the glomus cells were non-fluorescent, as was observed by comparing the fluorescence of unstained sections (Fig. 1) with sections stained with Toluidine Blue (Fig. 3). Such cells sometimes formed entirely non-fluorescent cell cords but they were also present in all cords mainly composed of fluorescent glomus cells. Most glomus cells exhibited a weak green FIF, while a minority of the cells exhibited a more intense FIF, and some of these showed a very intense, yellow fluorescence (Fig. 1). Although no spectral studies were carried out, it appeared that catecholamines, rather than 5-hydroxytryptamine, were responsible. The intensity of the FIF of most glomus cells was more intensely fluorescent than that of the nerve cell bodies but less intense than that of the SIF cells in the superior cervical ganglion, which often was seen near the carotid body in the same section. No nerve cell bodies were found amongst the cells of the carotid body but individual nerve cells or groups of them were occasionally seen on the surface of the body (nc in Fig. 1).

Table 1. Intensity of the formaldehyde-induced fluorescence in the glomus cells of the carotid body of adult rats.

Treatment	−	+	++	+++
Untreated controls	10	20	50	20
Hydrocortisone	0	20	40	40
Methylprednisolone	0	0	50	50

Scale: −, no; +, weak; ++, moderate; +++, intense fluorescence. The results are expressed as a percentage of the total number of glomus cells.

The other main cellular component of the carotid body, the sustentacular cells, surrounding the glomus cells as satellite cells, were essentially non-fluorescent. However, some thin leaf-like extensions of the sustentacular cells, tightly enveloping the glomus cells, exhibited a weak fluorescence of the same colour as that of the glomus cells.

Adrenergic nerve fibres formed a fluorescent network between the cords of the glomus cells. In fibres with their longitudinal axis in the plane of the section, several varicosities typical of sympathetic postganglionic terminal axon were seen. Such fluorescent fibres were observed both around the capillaries and in close contact with the cords of the glomus cells.

Changes induced by glucocorticoids

As is shown in Fig. 2, administration of methylprednisolone caused a clear increase in the fluorescence of the glomus cells, and the same applied to hydrocortisone-treatment (Table 1). In the carotid body of the rats given either of these two glucocorticoids, no non-fluorescent glomus cells were detected by comparison of the fluorescence specimens with the Toluidine Blue-stained sections (Figs. 2 & 4).

The colour of the FIF in most glomus cells of the glucocorticoid-treated rats was yellow, *i.e.* the same colour as that of the cells with a moderate or intense fluorescence in the controls. Fluorescent granules, presumably aggregates of small granular vesicles, sometimes visible at a high magnification with the light microscope in the cytoplasm of the glomus cells of the control rats, were rarely seen in the glomus cells of the injected rats, whose cytoplasm was evenly covered by the more intense FIF. The cords of the hormone-treated rats were of the same size as those in the controls.

Nerve fibres, sustentacular cells and other components of the carotid body did not show any distinguishable changes due to administration of either glucocorticoid.

Discussion

Since the formaldehyde-induced fluorescence of individual glomus cells varies from nothing to very intense in the carotid body of the adult rat, this organ is an interesting target for the study of changes in the catecholamine content. In the present study, administration of methylprednisolone and hydrocortisone caused a distinct increase in the over-all intensity of the catecholamine fluorescence. This observation suggests that glucocorticoids cause, in the *adult* carotid body, an increase in the formation and storage of catecholamines in previously existing glomus cells. Other, though less likely, possibilities are formation of new glomus cells by division or by differentiation from immature cells, mechanisms which may be responsible for the glucocorticoid-induced increase in the number of the SIF cells in the sympathetic ganglia of *newborn* rats (Eränkö & Eränkö, 1972).

Glucocorticoids do not cause any significant changes in the number or fluorescence intensity of the SIF cells in the sympathetic ganglia of adult rats (Eränkö & Eränkö, 1972). The increase in the catecholamine fluorescence of the glomus cells reported in the present study is indeed the first observation of its kind in adult animals. This raises the question of why do the glomus cells of the carotid body react to glucocorticoids, while the fluorescence microscopically very similar SIF cells in the sympathetic ganglia, which in newborn rats show a dramatic response, fail to show any change in adult rats. One explanation would be that the glomus cells remain even in adult rats at a more primitive stage which enables them to react to glucocorticoids with an increased formation of catecholamines, like the primitive SIF cells of the newborn rats (Eränkö & Eränkö, 1972). On the other hand, the differences in the fluorescence intensity of the glomus cells in the normal adult rats may reflect differences in their functional state, which may be connected with the formation, storage and depletion of catecholamines.

The chemoreceptor function of the carotid body is commonly accepted (for references, see Biscoe, 1971). However, the glomus cells may not necessarily be connected with that function (Zapata *et al.*, 1969). Indeed, it has been proposed that they are an accessory organ of internal secretion (Yates *et al.*, 1970). It is also possible that chemoreceptor and endocrine function can be connected in one and the same cell (Eränkö, 1972).

Although the SIF cells are more intensely fluorescent than most glomus cells, glomus cells closely resemble the SIF cells in shape. Evidence is also available for the common origin of these cells in the neural crest (Pearse *et al.*, 1973; Korkala, unpublished observations). Furthermore, the electron microscopical characteristics of the glomus cells and SIF cells are very similar, both containing numerous granular vesicles about 100 nm in

diameter (Matthews & Raisman, 1969; Yates *et al.*, 1970; Biscoe, 1971; Eränkö, 1972). In fact, the only fundamental difference is in their location relative to blood vessels: the glomus cells are always separated from the capillary wall by sustentacular cell processes (Hervonen & Korkala, 1972), while the SIF cells are in intimate contact with the basement membrane (Eränkö & Härkönen, 1965; Matthews & Raisman, 1969; Hervonen & Kanerva, 1972). While the location of the SIF cells near blood vessels provides the potential for endocrine function, as was pointed out by Eränkö & Härkönen (1965), and the glomus cells do not have such direct relation to blood vessels, this does not necessarily exclude the possibility of endocrine function of the carotid body glomus cells, whose morphological and histochemical properties strongly suggest such function (Yates *et al.*, 1970; Pearse *et al.*, 1973).

Glucocorticoids cause an increase in the number of granular vesicles in the cytoplasm of the SIF cells of newborn rats (Eränkö *et al.*, 1973). It would be of interest to know whether the glucocorticoid-induced increase in the fluorescence intensity of the glomus cells is similarly associated with an increase in the number of granular vesicles. Further studies are in progress to investigate this problem.

References

BISCOE, T. J. (1971). Carotid body: structure and function. *Physiol. Rev.* **51**, 437–95.

ERÄNKÖ, L. (1972). Histochemical and electron microscopical observations on catecholamines in developing sympathetic ganglion. *Acta inst. anat. (Helsinki), Suppl.* **5**, 1–30.

ERÄNKÖ, L. & ERÄNKÖ, O. (1972). Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. *Acta physiol. scand.* **84**, 125–33.

ERÄNKÖ, O. (1967). The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. R. microsc. Soc.* **87**, 259–76.

ERÄNKÖ, O. & ERÄNKÖ, L. (1971). Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. *Prog. Brain Res.* **34**, 39–51.

ERÄNKÖ, O., HEATH, J. & ERÄNKÖ, L. (1973). Effect of hydrocortisone on the ultrastructure of the small, granule-containing cells in the superior cervical ganglion of the newborn rat. *Experientia* **29**, 457–9.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1963). Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* **58**, 285–6.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1965). Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. *Acta physiol. scand.* **63**, 511–12.

HERVONEN, A. & KANERVA, L. (1972). Cell types of human fetal superior cervical ganglion. *Z. Anat. Entw.-Gesch.* **137**, 257–69.

HERVONEN, A., KANERVA, L., KORKALA, O. & PARTANEN, S. (1972). Effects of hypoxia and glucocorticoids on the histochemically demonstrable catecholamines of the newborn rat carotid body. *Acta physiol. scand.* **86**, 109–44.

HERVONEN, A. & KORKALA, O. (1972). Fine structure of the carotid body of the midterm human fetus. *Z. Anat. Entw.-Gesch.* **138**, 135–44.

KANERVA, L. (1972). Development, histochemistry and connections of the paracervical (Frankenhäuser) ganglion of the rat. A light and electron microscopic study. *Acta inst. anat. (Helsinki), Suppl.* **2**.

LEMPINEN, M. (1964). Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. *Acta physiol. scand.* **62**, Suppl. 231, 1–91.

MATTHEWS, M. R. & RAISMAN, G. (1969). The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. *J. Anat. (Lond.)* **105**, 255–82.

PEARSE, A. G. E., POLAK, J. M., ROST, F. W. D., FONTAINE, J., LE LIEVRE, C. & LE DOUARIN, N. (1973). Demonstration of the neural crest origin of type I (APUD) cells in the avian carotid body, using a cytochemical marker system. *Histochemie* **34,** 191–203.

PLOEM, J. S. (1971). The microscopic differentiation of colour of formaldehyde-induced fluorescence. *Prog. Brain Res.* **34,** 27–38.

ZAPATA, P., HESS, A., BLISS, E. L. & EYZAGUIRRE, C. (1969). Chemical, electron microscopic and physiological observations on the role of catecholamines in the carotid body. *Brain Res.* **14,** 473–96.

YATES, R. D., CHEN I-LI & DUNCAN, D. (1970). Effects of sinus nerve stimulation on carotid body glomus cells. *J. Cell Biol.* **46,** 544–52.

Z. Zellforsch. 146, 565—578 (1973)

Effect of Hydrocortisone on the Extra-adrenal Intensely Fluorescent Chromaffin and Non-chromaffin Cells in Stretch Preparations of the Newborn Rat

Marcello Costa*, Liisa Eränkö, and Olavi Eränkö**

Department of Anatomy, University of Helsinki, Helsinki, Finland
(Head: Prof. Olavi Eränkö, M. D., Ph. D.)

Received August 22, 1973

Summary. Air-dried stretch preparations were used to study adrenergic nerve fibres and catecholamine-containing cells with the formaldehyde-induced fluorescence method in the abdominal tissue block containing the para-aortic paraganglia, in the neurovascular structures of the cervical region including the carotid body, in the bladder, in the ileum, in the mesentery, in the vagus nerve and in the sympathetic ganglia of 5- or 15-day-old rats. The adrenergic nerves and the catecholamine-containing cells were well preserved and showed little or no diffusion of amines. While most intensely fluorescent cells of the main para-aortic body disappeared during the first two postnatal weeks, some such cells survived and they showed long, slender fluorescent processes. Administration of 20 mg/kg of hydrocortisone acetate daily for 5 days after birth caused a striking increase in the number and size of the clusters of the intensely fluorescent cells in the organ of Zuckerkandl, in the sympathetic ganglia and in the bladder, as well as an increase in the fluorescence intensity of the carotid body. In rats treated with hydrocortisone for 5 days and left to recover for 10 days an increased fluorescence was still observed. However, in the organ of Zuckerkandl the intensely fluorescent cells of hydrocortisone-treated 15-day-old rats showed less processes than those of the control rats of the same age.

Key words: Sympathetic neurons, Chromaffin cells, SIF cells (Newborn rat) — Development — Administration of hydrocortisone — Stretch preparations — Fluorescence microscopy.

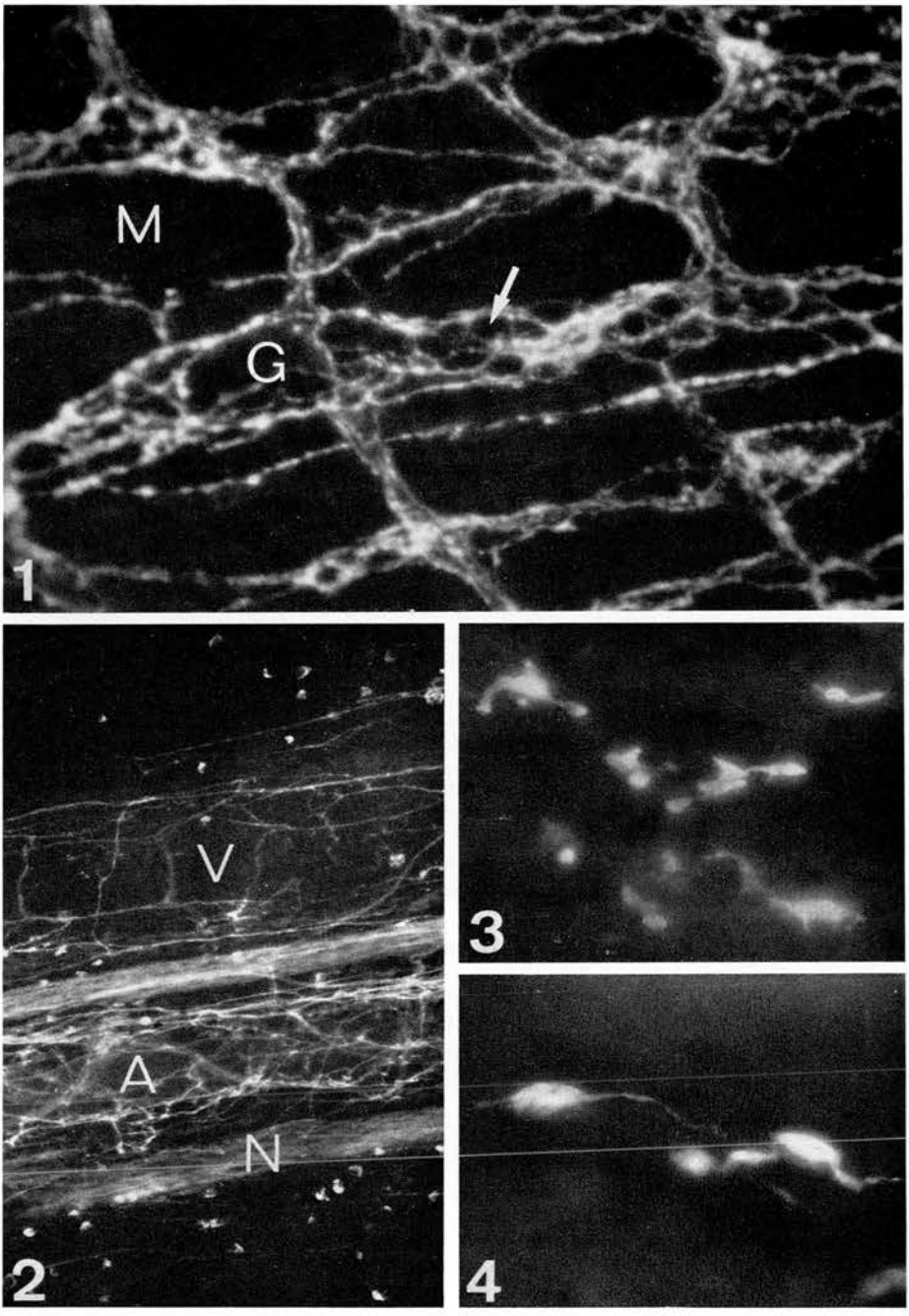
Introduction

The use of whole mount preparations has been found valuable in the study of the distribution of the adrenergic nerves and other catecholamine-containing structures in adult (Falck, 1962; Gabella and Costa, 1967; Costa and Gabella, 1971; Furness and Malmfors, 1971; Costa and Furness, 1973) and in newborn animals (Gabella and Costa, 1969). Observation of the extra-adrenal chromaffin tissue in whole mount preparations would be a suitable method to study the distribution and drug-induced changes in the number and size of the clusters of the extra-adrenal catecholamine-containing cells in the body, of which even serial sections would furnish an incomplete account.

---

* Sigrid Jusélius Foundation Research Fellow in the Department of Anatomy, University of Helsinki, April–July 1973. Present address: Department of Zoology, University of Melbourne, Parkville 3052, Victoria, Australia. Reprint requests to Professor O. Eränkö in the Helsinki address.

** The authors are greatly indebted to Dr. Daniela Costa for skilled help in the preparation of the illustrative material and the manuscript. — This work was supported by research grants from the Emil Aaltonen Foundation (to L. E.) and the Sigrid Jusélius Foundation (to O. E.).



Figs. 1—4. Air-dried stretch preparations showing the good preservation of catecholamine-containing structures with the whole mount technique in developing animals

Fig. 1. Auerbach's plexus in the ileum of a 5-day-old rat. In the ganglia (*G*) the black (non-fluorescent) ganglion cells (arrow) are surrounded by fluorescent fibres. Both longitudinal and circular muscle layers (*M*) were included in the preparation. ×290

Hydrocortisone has been shown to produce hyperplasia of the extra-adrenal chromaffin tissue in the rat (Lempinen, 1964), which has been confirmed by fluorescence histochemistry and extended to the small, non-chromaffin, intensely fluorescent cells in sympathetic ganglia (Eränkö and Eränkö, 1972; Ciaranello *et al.*, 1973).

The present investigation was undertaken to study the effect of hydrocortisone in the extra-adrenal catecholamine-containing cells on newborn rats in whole mount preparations of a variety of tissues.

Materials and Methods

Four litters of newborn rats, descendants of the Sprague-Dawley strain were used. Two litters were injected subcutaneously with 20 mg/kg. Body weight of hydrocortisone acetate (N. V. Organon) daily for 5 days. The other two litters served as controls. The rats of one injected and one control litter were sacrificed on the 5th day after birth five hours after the last injection, the rats of the other two litters on the 15th day after birth 10 days after the last injection. The rats were killed by cutting the spinal cord and the aorta at the level of the heart.

Immediately after killing, the tissues were removed and put in physiological saline solution containing 0.1% of glucose. The solution was kept in beakers in crushed ice. The whole retroperitoneal tissue block from the level of the renal arteries to the iliac bifurcation, the ileum, the mesentery, the bladder, the neurovascular structures of the cervical region including the carotid bifurcation, the carotid body, the vagus nerve and the sympathetic cervical chain were taken. The tissues were quickly dried with blotting paper, stretched on slides and air-dried over phosphorus pentoxide for 1 hour (for details of the technique see Costa and Gabella, 1971; Costa and Furness, 1973). The slides were then exposed to formaldehyde vapour for 30 min./at 50°C and subsequently for 1 hour at 80°C. The vapour was generated from paraformaldehyde powder equilibrated with 60% relative air humidity. The preparations were mounted in Entellan and their fluorescence was examined and photographed using the Ortholux microscope (E. Leitz, Wetzlar) with transmitted exciting light from the Osram HBO 200 lamp fitted with the following filters: 3 mm BG 38, 3 mm BG 12, 3 mm BG 3 and the Leitz K 510 filter for quenching the ultraviolet light.

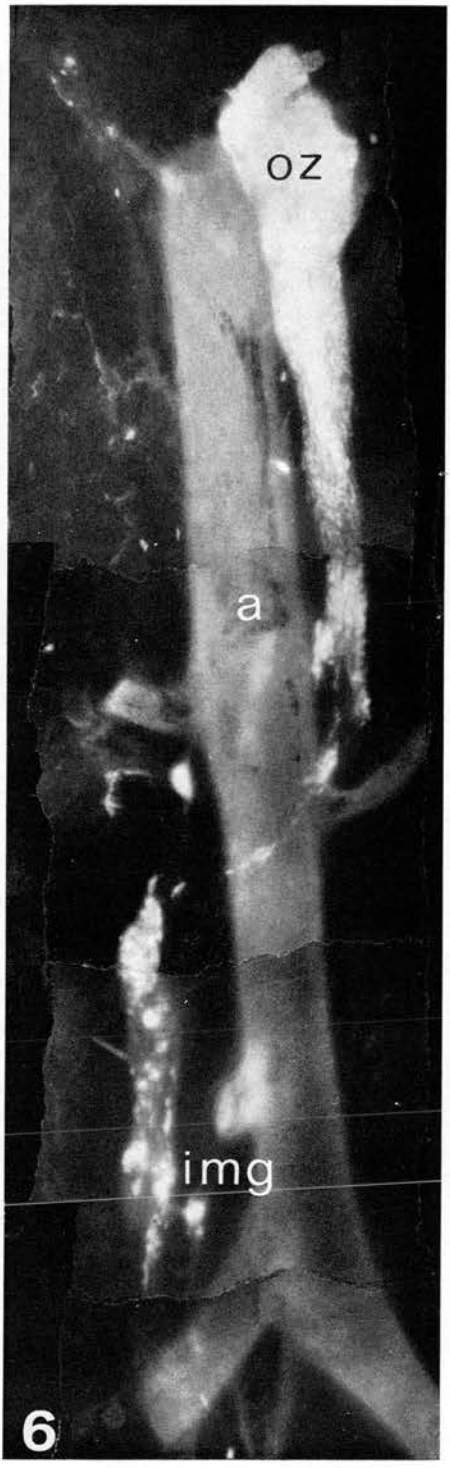
Results

General Observations. Whole mount preparations of 5- and 15-day-old control rats are shown in Figs. 1–4. Under optimum conditions the adrenergic nerves and the catecholamine-containing cells were well preserved with little or no diffusion of amines. The nerve fibres of the Auerbach plexus showed distinct varicosities (Fig. 1). The nerve nets around the mesenterial veins and arteries were demonstrated in great detail (Fig. 2). Fluorescent cells in the para-aortic region were sharply delineated (Figs. 3, 4).

Fig. 2. Mesentery of a 5-day-old rat. A mesenteric artery (*A*) is shown with a perivascular plexus of adrenergic fibres. The accompanying vein (*V*) shows a sparser perivascular plexus. The paravascular nerve trunks (*N*) running parallel to the blood vessels are composed of smooth fibres. ×226

Fig. 3. Intensely fluorescent cells in the para-aortic region of a 5-day-old rat. ×362

Fig. 4. Intensely fluorescent cells in the para-aortic region of a 15-day-old rat. Note the fluorescent processes arising from small clusters of these cells. ×362



Figs. 5 and 6

Abdominal Fluorescent Tissues. In the retroperitoneal region of the 5-day-old control rats (Fig. 5) the main para-aortic body, the Organ of Zuckerandl (OZ) appeared as a discrete fluorescent mass adjacent to the aorta. Smaller clusters of fluorescent cells were present along the first tract of the mesenteric nerves, which were included in the preparation, in the retroperitoneal fat, in the superior and inferior mesenteric ganglia and in the small ganglia scattered along the hypogastric nerves (Fig. 9). At higher magnification (Figs. 3, 7) the OZ was seen to be composed of small clusters of, or single, intensely fluorescent cells, which were elongated and without long processes.

A striking increase in the number and size of the clusters of the intensely fluorescent cells was observed in the hydrocortisone-treated rats (compare Figs. 5 and 6). The OZ appeared as a dense, intensely fluorescent body. The fluorescent cells were so densely packed (Fig. 8) as compared with the control (Fig. 7) that individual cells could not be recognized. The organ was richly vascularized. The number and the size of the small clusters of fluorescent cells in the retroperitoneal fat and along the mesenteric nerves were increased. Likewise, the number of intensely fluorescent cells in the prevertebral sympathetic ganglia was increased (Figs. 6, 10) as compared to the controls (Figs. 5, 9).

In the whole mounts of the para-aortic region of the 15-day-old control animals only small clusters of fluorescent cells were visible in the region of the OZ (Fig. 11), which is known to undergo degeneration soon after birth in the rat (see Lempinen, 1964). The surviving fluorescent cells of the normal 15-day-old rats showed more often processes (Figs. 4, 13) than those of the 5-day-old control animals (Fig. 3). A few intensely fluorescent cells were observed in all prevertebral ganglia, and small clusters of intensely fluorescent cells were seen scattered in the retroperitoneal tissue.

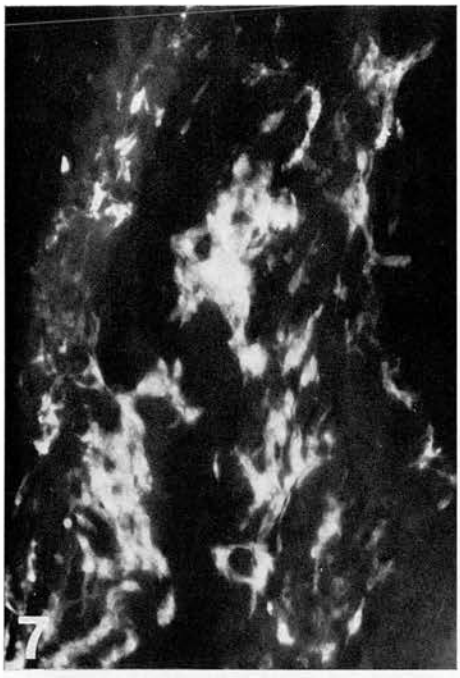
In the rats treated with hydrocortisone for 5 days and left to recover for 10 days the fluorescence was still increased in the retroperitoneal region. The OZ (Figs. 12, 14) showed a striking difference from that of the corresponding controls (Figs. 11, 13), although it appeared less prominent and compact than that of the 5-day-old rats killed 5 hours after the last hydrocortisone injection (Figs. 6, 8). The processes of the cells in the 15-day-old hydrocortisone-treated rats were less prominent (Fig. 14) than those of the fluorescent cells of the corresponding control rats (Figs. 4, 13).

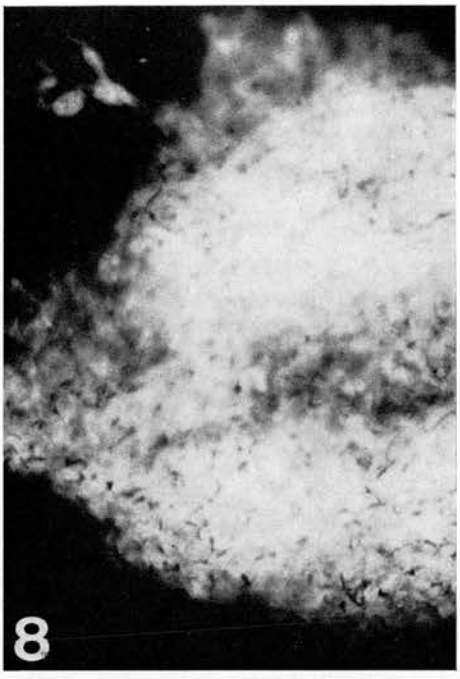
Also in the abdominal sympathetic ganglia the number of intensely fluorescent cells was increased in hydrocortisone-treated 15-day-old rats.

Bladder. In the 5-day-old-control rats a large number (about 150 in the whole bladder) of small fluorescent cells, isolated or in small clusters, were found in the muscle layer of the bladder. Following hydrocortisone treatment the intensity of the fluorescence was markedly increased, so that the nuclei were not visible while the number of the cells was not significantly altered.

Figs. 5 and 6. Reconstruction photographs of air-dried stretch preparations of the whole retroperitoneal region of 5-day-old control (Fig. 5) and hydrocortisone-treated (Fig. 6) rats. The whole organ of Zuckerkandl (*OZ*) is shown in the figures adjacent to the aorta (*a*). In the hydrocortisone-treated rat the OZ is more densely packed with intensely fluorescent cells.

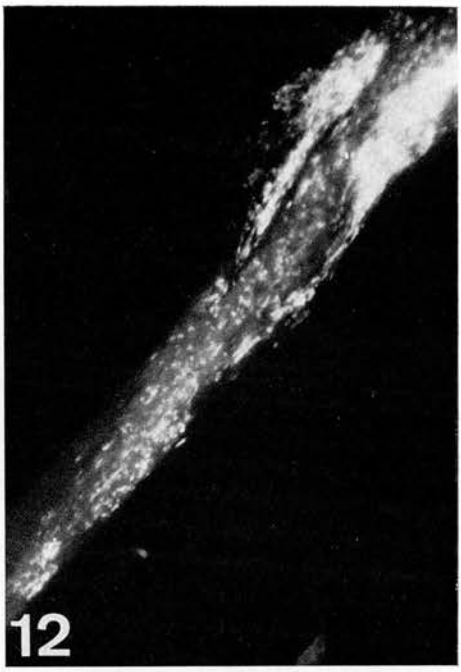
Similar fluorescence increase can be seen in the inferior mesenteric ganglion. (*img*) ×28



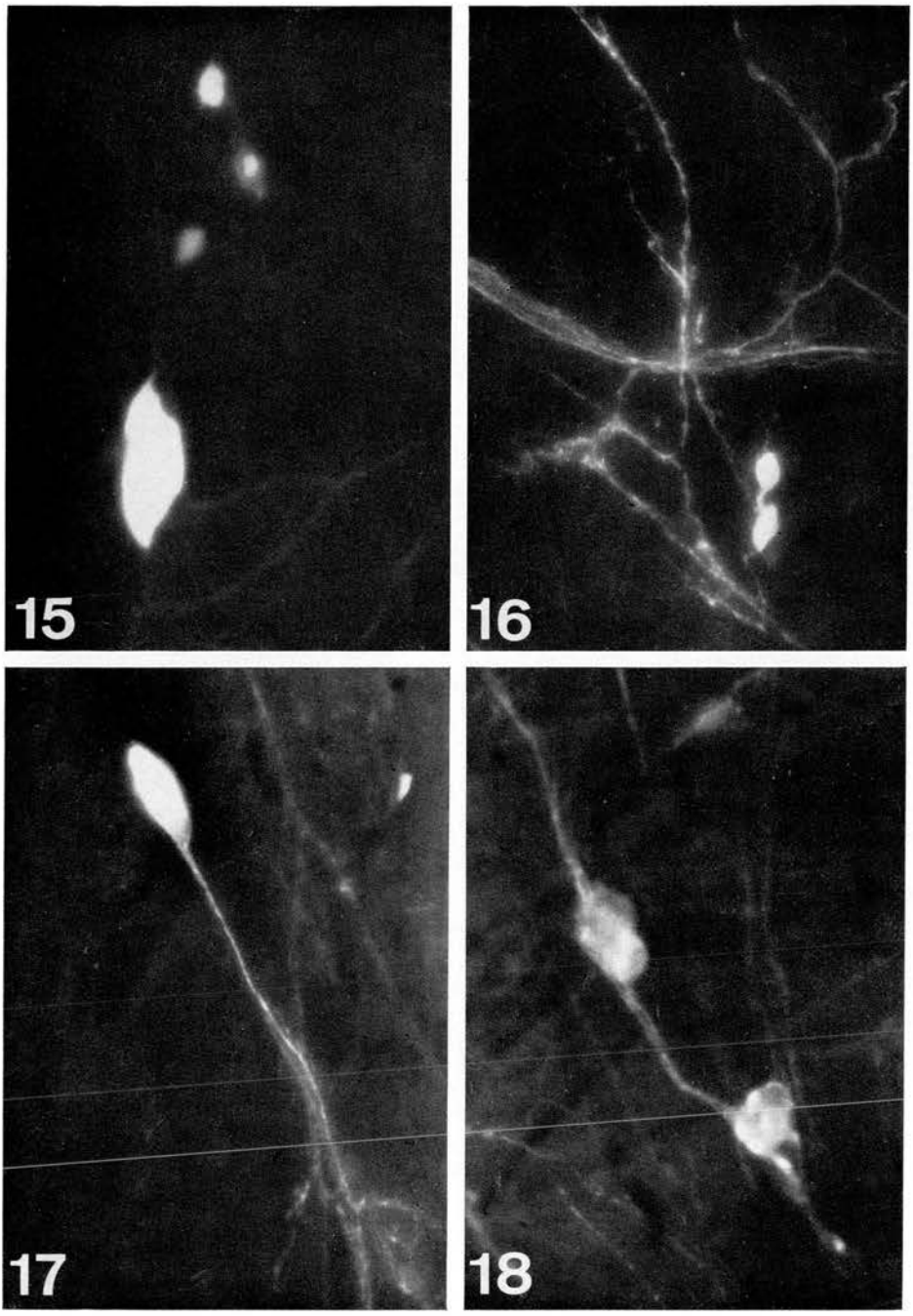


Figs. 7 and 8. Higher magnification of Organ of Zuckerkandl shown in Figs. 5 and 6. The number of the intensely fluorescent extra-adrenal chromaffin cells is markedly increased in the hydrocortisone-treated 5-day-old rat. $\times 226$

Figs. 9 and 10. Small ganglia along the hypogastric nerves of 5-day-old rats. In the control ganglion (Fig. 9) there are only a few intensely fluorescent cells, while a large number of such cells are visible in the hydrocortisone-treated rat (Fig. 10). The weaker diffuse fluorescence in the ganglia is due to adrenergic neurons. $\times 226$



Figs. 11—14. Air-dried stretch preparations of the retroperitoneal region of 15-day-old rats. Fig. 11 shows the Organ of Zuckerkandl (*OZ*) of the control rat, Fig. 12 that of a hydrocortisone-treated rat $\times 75$. Figs. 13 and 14 are details of Figs. 11 and 12 at a higher magnification. There are numerous processes in the intensely fluorescent cells of the OZ of the control rat (Fig. 13). In the hydrocortisone-treated rats (Fig. 14) the processes are less developed. $\times 226$



Figs. 15—18. Air-dried stretch preparations of the bladder of 15-day-old rats

Fig. 15. Clusters of intensely fluorescent cells in the bladder wall. Because of the intense cytoplasmic fluorescence covering the nuclei, individual cells cannot be seen. ×226

In the 15-day-old control rats but a few weakly fluorescent cells with similar characteristics were found in the bladder musculature. In the hydrocortisone-treated 15-day-old rats numerous small fluorescent cells were present, usually in clusters, and the intensity of fluorescence of these cells was very intense as compared with the controls. Figs. 15 and 16 show some single intensely fluorescent cells and a cluster of them in the wall of the bladder of 15-day-old rats treated with hydrocortisone. About 6 fluorescent nerve cell bodies were observed in the whole bladder (Figs. 17, 18). The small number of nerve cells makes it understandable that they were not observed in a previous study on the rat bladder (El-Badawi and Schenk, 1969).

Cervical Neurovascular Structures. The carotid body of the control rats appeared in the whole-mount preparation of the cervical tissue block as a moderately fluorescent mass (Fig. 19), which was located at the carotid bifurcation in close association with the superior cervical ganglion. Because of its thickness it was not possible to stretch this ganglion flat. In the 15-day-old hydrocortisone-treated animals the carotid body still showed a markedly increased fluorescence intensity (Fig. 20), in agreement with the previous observation by Hervonen *et al.* (1972) made using sectioned material.

A few intensely fluorescent cells, single or forming small clusters, were observed embedded or along the vagus nerve in the cervical region. In the hydrocortisone-treated rats, a larger number of clusters, which were also of larger size, were observed along the vagus nerve.

Discussion

The present work shows that the extra-adrenal catecholamine-containing tissue can be easily demonstrated in newborn animals in air-dried whole-mount preparations using the formaldehyde-induced fluorescence method. This technique, which has been used succesfully on a variety of organs to demonstrate adrenergic fibres (see the introduction), has been shown to be useful in the study of the distribution of extra-adrenal chromaffin tissue in the abdominal and pelvic region of adult guinea-pigs (Costa and Furness, 1973). In the present study, examination of whole mounts of stretched tissues from newborn rats allowed reliable comparison of the amount and the distribution of catecholamine-containing cells in young hydrocortisone-treated and control rats. The discussion of the present work is limited to previous observations made on the rat only, because this species is unique in so far that there is a marked postnatal involution of catecholamine-storing tissues, which turn non-chromaffin (Lempinen, 1964; Eränkö and Eränkö, 1973) in contrast to those of other species (Coupland, 1965).

Fig. 16. Fluorescent varicose nerve fibres in the trigonum area of the bladder. Two intensely fluorescent cells are also visible. Magnification ×265

Fig. 17. An intensely fluorescent cell in the bladder wall from whose cytoplasm emerges a fluorescent process, probably a nerve fibre. ×362

Fig. 18. Fluorescent nerve cell bodies in the bladder wall. The less intensely fluorescent nucleus is visible in the lower cell. ×362

Figs. 19 and 20. Air-dried stretch preparations of the carotid body of 15-day-old rats. The intensity of the fluorescence is increased in the hydrocortisone-treated rat (Fig. 20), as compared with the control rat (Fig. 19). Single cells cannot be identified in the thick carotid body. The small dark spots on the bodies are blood vessels which richly supply this organ. Note that Fig. 20 had to be exposed much less than Fig. 19 owing to the very intense fluorescence, as is shown by the darker background. In reality the difference between the fluorescence intensities of these two bodies was more pronounced. ×226

The marked reduction observed in this study during the two postnatal weeks in the number of the intensely fluorescent cells of the main para-aortic body, the OZ, parallels the loss of the chromaffin reaction from, and the disintegration of, the para-aortic body of the rat, as was previously described by Lempinen (1964).

The dramatic increase which we observed in the number of the intensely fluorescent cells in the para-aortic region following treatment with hydrocortisone confirms the observations by Lempinen (1964), who used the chromaffin reaction, and those by Eränkö and Eränkö (1972) and Ciaranello *et al.* (1973), who used the formaldehyde-induced fluorescence method. In the present study the hyperplasia of the extra-adrenal chromaffin tissue lasted for several days after cessation of the hydrocortisone treatment, also confirming previous results (Lempinen, 1964; Ciaranello *et al.*, 1973).

Our study further demonstrated in stretch preparations intensely fluorescent cells in the retroperitoneal tissue, along the mesenteric nerves and in other sites of the rat described previously by other authors in sections, such as the inferior mesenteric ganglion (Van Orden *et al.*, 1970), the hypogastric nerves (Owman and Sjöstrand, 1965), the cervical vagus nerve (Gabella and Costa, 1968). The fluores-

cence microscopic appearance of these cells was similar to that of the intensely fluorescent cells described in the superior cervical ganglion of the rat by Eränkö and Härkönen (1963) and studied in more detail by several authors in adult and newborn rats (Norberg and Hamberger, 1964; Eränkö and Härkönen, 1965; Norberg *et al.*, 1966; Taxi *et al.*, 1969; Van Orden *et al.*, 1970; Björklund *et al.*, 1970; Eränkö and Eränkö, 1971; Eränkö, 1972a–b; Ciaranello *et al.*, 1973). Similar cells were also described in the rat heart (Jacobowitz, 1967; Ehinger *et al.*, 1968) and in the paracervical ganglion of the rat uterus (Kanerva, 1972).

Electron microscopic investigations have shown that these cells have common ultrastructural features, such as the presence in the cytoplasm of granular vesicles resembling those of the medullary cells (Coupland, 1965; Elfvin, 1965; Mopper, 1966). In the superior cervical ganglion of the newborn rat, the diameter of the granular vesicles of the small intensely fluorescent cells is 90–150 nm (Eränkö, 1972b), in the adult variable figures have been reported: 80–100 nm (Hökfelt, 1969), 70–100 nm (Taxi *et al.*, 1969), 100–150 nm with some larger vesicles of 200–400 nm (Sigrist *et al.*, 1966 and 1968), 65–120 nm (Matthews and Raisman, 1969), average 140 nm (Williams and Palay, 1969), 150–250 nm (Van Orden *et al.*, 1970) and 40–140 nm (Tamarind and Quillam, 1971). Granular vesicles have also been reported in the small intensely fluorescent cells of the inferior mesenteric ganglion (Van Orden *et al.*, 1970; 150–250 nm), in the paracervical ganglion of the newborn and adult female rat (Kanerva, 1972; 80–200 nm and 80–140 nm, respectively, with some granules of 200–300 nm in the adult rat). In an electron microscopic study on the OZ, the main para-aortic body, in the newborn rat, a range of 50–200 nm has been reported for the diameter of the granular vesicles (Mascorro and Yates, 1970). This suggests that the intensely fluorescent cells demonstrated in the rat all have granular vesicles of approximately same appearance and size range.

The question then arises whether the chromaffin cells, which are intensely fluorescent in the para-aortic region of normal newborn rats and the chromaffin cells which have been reported in the normally non-chromaffin sympathetic nerves and ganglia after hydrocortisone treatment (Lempinen, 1964) are of the same type as the non-chromaffin small intensely fluorescent (SIF) cells found in the sympathetic ganglia of the rat (Eränkö and Eränkö, 1971). Glucocorticoids induce the appearance of the phenylethanolamine-N-methyltransferase both in the para-aortic chromaffin tissue (Axelrod, 1966; Roffi and Margolis, 1966) and in the superior cervical ganglion (Ciaranello *et al.*, 1973). It is unlikely that the new, intensely fluorescent chromaffin cells induced by glucocorticoids in the superior cervical ganglion (Lempinen, 1964; Eränkö and Eränkö, 1972) are modified adrenergic neurons, as was suggested by Ciaranello *et al.* (1973). Electron microscopic studies (Eränkö *et al.*, 1973) have in fact demonstrated that at least many hydrocortisone-induced small intensely fluorescent cells have the characteristic large granular vesicles in the cytoplasm. Whether hydrocortisone induces an increase in the tubular organelles and the endoplasmic reticulum of the cytoplasm remains to be seen. However, even in the fluorescence microscope the adrenergic neurons of the superior cervical ganglion of newborn rats which show an increased fluorescence after hydrocortisone treatment (Costa *et al.*, 1973) can be easily differentiated from the small intensely fluorescent cells (Eränkö, 1972a).

It is likely that the differences between the intensely fluorescent chromaffin and non-chromaffin cells in the rat are due to differences in the amount of catecholamines contained by the cytoplasm and the diameter of the granular vesicles Eränkö and Eränkö, 1973). Several observations support this view: (1) The sensitivity of the chromaffin reaction for the detection of catecholamines by light microscopy is poor and therefore high concentration is required. (2) The size of the granular vesicles in the non-chromaffin fluorescent cells of the rat are smaller than those of the medullary chromaffin cells (Matthews and Raisman, 1969). (3) A chromaffin reaction appears in the cells following glucocorticoid treatment (Lempinen, 1964). (4) Hydrocortisone increases the catecholamine content of the extra-adrenal catecholamine-containing cells (Roffi, 1965; Eränkö *et al.*, 1966). (5) Hydrocortisone causes an increase in the number and electron density of the granular vesicles in the SIF cells *in vivo* (Eränkö *et al.*, 1973), as well as an increase in their diameter in cultures of sympathetic ganglia (Eränkö *et al.*, 1972).

The involution of the extra-adrenal chromaffin tissue was in this study associated with morphological changes as well: the few intensely fluorescent cells which survived the normal involution of the abdominal paraganglia after birth showed processes similar to those of the small intensely fluorescent cells of the adult sympathetic ganglia, while the numerous intensely fluorescent cells in the hydrocortisone-treated rats showed no or few processes. Similar observations have been made on the para-aortic tissues of the newborn rabbit (Costa, unpublished results). These observations and the fact that even adrenal medullary cells can grow processes resembling adrenergic nerve fibres (Olson, 1970) suggest that all catecholamine-rich cells may be affected by environmental factors so that they may develop into functionally and morphologically different types of related cells.

It is possible that there are several types of intensely fluorescent granule-containing cells in the rat, as well as in other species. However, the differentiation of such cell types cannot be based on the chromaffinity or the lack of it or merely on the presence of the fluorescence induced by formaldehyde vapour. More detailed information on the nature of the amines and their mode of storage in these cells, on their nervous connections and humoral sensitivity, as well as on the function of these cells is required before new classification can be attempted.

References

Axelrod, J.: Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. Pharmacol. Rev. **18**, 95–113 (1966)

Björklund, A., Cegrell, L., Falck, B., Ritzen, M., Rosengren, E.: Dopamine-containing cells in sympathetic ganglia. Acta physiol. scand. **78**, 334–338 (1970)

Ciaranello, R.D., Jacobowitz, D., Axelrod, J.: Effect of dexamethasone on phenyl-ethanolamine N-methyltransferase in chromaffin tissue of the neonatal rat. J. Neurochem. **20**, 799–805 (1973)

Costa, M., Eränkö, O., Eränkö, L.: Hydrocortisone-induced increase in the histochemically demonstrable catecholamine content of the sympathetic neurons of the newborn rat. Brain Res. (in press) (1973)

Costa, M., Furness, J.B.: Observations on the anatomy and amine histochemistry of the nerves and ganglia which supply the pelvic viscera and on the associated chromaffin tissue in the guinea pig. Z. Anat. Entwickl.-Gesch. **140**, 85–108 (1973)

Costa, M., Gabella, G.: Adrenergic innervation of the alimentary canal. Z. Zellforsch. **122**, 357–377 (1971)

Coupland, R. E.: Electron microscopic observations on the structure of the rat adrenal medulla. J. Anat. (Lond.) **99**, 231–254 (1965)

Ehinger, B., Falck, B., Persson, H., Sporring, B.: Adrenergic and cholinesterase-containing neurons of the heart. Histochemie **16**, 197–205 (1968)

El-Badawi, A., Schenk, E.: Dual innervation of the mammalian urinary bladder. Amer. J. Anat. **119**, 405–428 (1966)

Elfvin, L. G.: The fine structure of the cell surface of chromaffin cells in the rat adrenal medulla. J. Ultrastruct. Res. **12**, 263–286 (1965)

Eränkö, L.: Postnatal development of histochemically demonstrable catecholamines in the superior cervical ganglion of the rat. Histochem. J. **4**, 225–236 (1972a)

Eränkö, L.: Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. Brain Res. **46**, 159–175 (1972b)

Eränkö, L., Eränkö, O.: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat. Acta physiol. scand. **84**, 125–133 (1972)

Eränkö, O., Eränkö, L.: Small, intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. In: Histochemistry of nervous transmission. Progr. Brain Res. **34**, 39–51 (1971)

Eränkö, O., Eränkö, L.: Small intensely fluorescent (SIF) cells in vivo and in vitro. In: Front. Catecholamine Res. (in press) (1973)

Eränkö, O., Härkönen, M.: Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. Acta physiol. scand. **58**, 285–286 (1963)

Eränkö, O., Härkönen, M.: Monoamine-containing small cells in the superior cervical ganglion of the rat and an organ composed of them. Acta physiol. scand. **63**, 511–512 (1965)

Eränkö, O., Heath, J. W., Eränkö, L.: Effect of hydrocortisone on the ultrastructure of the small, intensely fluorescent, granule-containing cells in cultures of sympathetic ganglia of newborn rats. Z. Zellforsch. **134**, 297–310 (1972)

Eränkö, O., Heath, J.W., Eränkö, L.: Effect of hydrocortisone on the ultrastructure of the small, granule-containing cells in the superior cervical ganglion of the newborn rat. Experientia (Basel) **29**, 457–459 (1973)

Eränkö, O., Lempinen, M., Räisänen, L.: Adrenaline and noradrenaline in the organ of Zuckerkandl and adrenals of newborn rats treated with hydrocortisone. Acta physiol. scand. **66**, 253–254 (1966)

Falck, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. **56**, Suppl. 197 (1962)

Furness, J.B., Malmfors, T.: Aspects of the arrangement of the adrenergic innervation in guinea-pigs as revealed by the fluorescence histochemical method applied to stretched, air dried preparations. Histochemie **25**, 297–309 (1971)

Gabella, G., Costa, M.: Le fibre adrenergiche nel canale alimentare. G. Accad. Med. Torino **130**, 198–217 (1967)

Gabella, G., Costa, M.: Sulla presenza de particolari cellulare a catecholamine (S.I.F. cells) cellule lungo il nervo vago. Boll. Soc. Ital. Biol. Sperim. **44**, 1656–1657 (1968)

Gabella, G., Costa, M.: Distribution of adrenergic fibres in the small intestine of the newborn guinea-pig. Panminerva med. **2**, 407–409 (1969)

Hökfelt, T.: Distribution of noradrenaline storing particles in peripheral adrenergic neurons as revealed by electron microscopy. Acta physiol. scand. **76**, 427–440 (1969)

Jacobowitz, D.: Histochemical studies of the relationship of chromaffin cells and adrenergic nerve fibres to the cardiac ganglia of several species. J. Pharmacol. exp. Ther. **158**, 227–240 (1967)

Kanerva, L.: Development, histochemistry and connections of the paracervical (Frankenhäuser) ganglia of the rat uterus. Acta Inst. anat. Univ. Helsinki., Suppl. **2** (1972)

Lempinen, M.: Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. Acta physiol. scand. **62**, Suppl. 231 (1964)

Mascorro, J.A., Yates, R.: Microscopic observations on abdominal sympathetic paraganglia. Texas Rep. Biol. Med. **28**, 59–68 (1970)

Matthews, M. R., Raisman, G.: The ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. J. Anat. (Lond.) **105**, 255–282 (1969)

Moppert, J.: Zur Ultrastruktur der phaeochromen Zellen im Nebennierenmark der Ratte. Z. Zellforsch. **74**, 32–44 (1966)

Norberg, K. A., Hamberger, B.: The sympathetic adrenergic neuron. Acta physiol. scand. **63**, Suppl. 238 (1964)

Norberg, K. A., Ritzen, M., Ungerstedt, U.: Histochemical studies on a special catecholamine-containing cell type in sympathetic ganglia. Acta physiol. scand. **67**, 260–270 (1966)

Olson, L.: Fluorescence histochemical evidence for axonal growth and secretion from transplanted adrenal medullary tissue. Histochemie **22**, 1–7 (1970)

Orden, L. S. van III, Burke, J. P., Geyer, M., Lodoen, F. V.: Localization of depletion-sensitive and depletion-resistant norepinephrine storage sites in autonomic ganglia. J. Pharmacol. exp. Ther. **174**, 56–71 (1970)

Owman, C., Sjöstrand, N.O.: Short adrenergic neurons and catecholamine-containing cells in vas deferens and accessory male genital glands of different mammals. Z. Zellforsch. **66**, 300–320 (1965)

Roffi, J.: Influence de la corticosurrénale sur le contenue en adrénaline et noradrénaline de la surrénale, chez le foetus de Rat. C. R. Acad. Sci. (Paris) **260**, 1267–1270 (1965)

Roffi, J., Margolis, F.: Synthèse d'adrénaline dans le tissue chromaffine extrasurrénalien, chez le Rat nouveau-né, sous l'effect de l'hydrocortisone ou de la corticostimuline. C. R. Acad. Sci. (Paris) **263**, 1496–1499 (1966)

Siegrist, G., Dolivo, M., Dunant, Y., Foroglou-Kerameus, C., Ribaupierre, F. de, Rouiller, C.: Ultrastructure and function of the chromaffin cells in the superior cervical ganglion of the rat. J. Ultrastruct. Res. **25**, 381–407 (1968)

Siegrist, G., Ribaupierre, F. de, Dolivo, M., Rouiller, C.: Les cellules chromaffines des ganglions cervical superieurs du Rat. J. Microscopie **5**, 791–794 (1966)

Tamarind, D. L., Quilliam, J. P.: Synaptic organisation and other ultrastructural features of the supérior cervical ganglion of the rat, kitten and rabbit. Micron **2**, 204–234 (1971)

Taxi, J., Goutron, J., L'Hermite, P.: Données ultrastructurales sur une éventuelle modulation adrénergique de l'activité du ganglion cervical supérieur du Rat. C. R. Acad. Sci. (Paris) **269**, 1281–1284 (1969)

Williams, T. H., Palay, S. L.: Ultrastructure of the small neurons in the superior cervical ganglion. Brain Res. **15**, 17–34 (1969)

Dr. M. Costa
Dept. of Zoology
University of Melbourne
Victoria
Australia

Histochemical Journal, **6** (1974), 35–53

Histochemical correlates of cold-induced trans-synaptic induction in the rat superior cervical ganglion

MARCELLO COSTA* *and* OLAVI ERÄNKÖ

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

Received 16 July 1973

Synopsis. The effect of prolonged pre-ganglionic activity induced by exposure to cold (5°C) was studied in intact and decentralized superior cervical ganglia of rats. Intact and decentralized ganglia of rats kept at room temperature served as controls. Catecholamines were demonstrated histochemically using the formaldehyde-induced fluorescence method. The intensity of the fluorescence of the nerve cell bodies was estimated both visually and photometrically.

Decentralization in itself had no effect on the intensity of the cell bodies for up to 16 days. Exposure to cold had no effect on the decentralized ganglia but caused a marked increase in the fluorescence intensity of some nerve cells of the intact ganglia, indicating that the increased fluorescence was mediated by the pre-ganglionic nerves. The increase lasted for the whole 16-day-length of exposure to cold.

It is suggested that the observed change in the fluorescence intensity reflects an increase of the enzymes tyrosine hydroxylase and dopamine-β-hydroxylase, and thus represents a histochemical correlate of trans-synaptic induction.

Introduction

Previous studies have shown that a prolonged increase in the pre-ganglionic activity results in an induction of the enzymes tyrosine hydroxylase and dopamine hydroxylase in sympathetic ganglia (Mueller *et al.*, 1969*a*; Thoenen *et al.*, 1969*a*; Molinoff *et al.*, 1970; Thoenen, 1970; Axelrod, 1972). The neurally mediated induction of these enzymes is associated with an increase of noradrenaline synthesis in sympathetic ganglia (Gordon *et al.*, 1966; Thoenen, 1972), but the associated changes in the storage level of noradrenaline have not been investigated.

A change in the fluorescence intensity of the adrenergic nerve cell bodies after prolonged increase of pre-synaptic activity would represent a histochemical correlate of the

* Sigrid Jusélius Foundation Research Fellow in the Department of Anatomy, University of Helsinki, April–July 1973.
Present Address: Department of Zoology, University of Melbourne, Parkville 3052, Victoria, Australia.

trans-synaptic induction. With the aid of a histochemical method it would then be possible to localize and count the adrenergic neurons in a ganglion which are affected by an increased pre-ganglionic activity.

The present work was undertaken to study the effect of prolonged pre-ganglionic activity, induced by exposure to cold, on the intensity of the formaldehyde-induced fluorescence in the adrenergic cell bodies of the superior cervical ganglion of the rat.

Material and methods

EXPERIMENTAL

Adult male albino rats, descendants of the Sprague-Dawley strain, were used. The right superior cervical ganglion was decentralized in twenty-four animals and the outcome of the denervation was checked by the ptosis in the right eye. After one day of recovery, one group was placed in a cold room kept at 5°C and another group was kept at 22°C. Five and 16 days later, animals were taken from both groups and killed by cutting the vertebral column and aorta with scissors under ether anaesthesia. The fluorescence induced by formaldehyde vapour (see Eränkö, 1967) was used to demonstrate catecholamines in the adrenergic neurons. The superior cervical ganglia were quickly removed, placed on copper grids and frozen by immersion in propane cooled with liquid nitrogen. Freeze-drying was carried out for 7 days at −45°C. The tissues were then exposed to formaldehyde vapour generated from paraformaldehyde, which had been previously equilibrated at 60% relative humidity for 30 min at 50°C followed by a further hour at 80°C, and embedded in paraffin wax under vacuum. Denervated and intact ganglia taken from each group of animals were treated in the same reaction vessel to minimize variations in the histochemical procedure. Pairs of ganglia, decentralized and intact, from rats kept at 22°C and those exposed to 5°C, were embedded in the same paraffin block to facilitate comparison.

Serial sections of the ganglia were cut at 12 μm and then were mounted in Entellan. For the fluorescence microscopic examination of the catecholamines, an Ortholux microscope (E. Leitz, Wetzlar) was used. It was fitted with an epi-illuminator (Ploem, 1967) and the following filters (Schott & Gen) after the Osram HBO 200 lamp: 3 mm BG 38, 3 mm BG 3, TAL 405, K 470.

ESTIMATION OF THE FLUORESCENCE INTENSITY

Two methods, visual and photometric, were used.

Photometric estimation

The intensity of the fluorescence was measured using the Leitz Orthomat camera. The spot field of the camera measured a field of diameter 30 μm, usually covering the whole cell body. An adjustable field diaphragm was used and the illuminated field reduced to that size in order to avoid photodecomposition of cells outside the measuring area. Sections which had not been exposed previously to u.v. light were used for the measurements. The intensity of one hundred cells was measured in each ganglia. These were all the cells that appeared in the field when the stage was moved parallel with the longitudinal axis of the ganglion from sections cut through the centre of the ganglion. By this linear cell sampling method, errors of subjective selection were eliminated and a sample

of cells reasonably representative of the ganglion population was obtained. The intensity was expressed in units obtained by dividing by 100 the time of exposure. A pair of decentralized and intact ganglia embedded in the same block and cut with the same stroke were measured with the same automatic camera setting. The setting was so chosen that the time of exposure of clusters of small intensely fluorescent cells was about 2 sec, and that of the non-fluorescent nerve cell bodies longer than 14 sec. No adjustment of the camera setting was required even for measurements of ganglia of different animals.

The reproducibility and the simplicity of the method were satisfactory but it also had limitations. Thus, weakly fluorescent cells suffered from a longer photodecomposition than intensely fluorescent ones; the field size was constant, although the diameter of the cell bodies was variable; fluorescent structures, other than the nerve cell bodies, were sometimes included in the field measured; the non-specific fluorescence not due to the specific fluorophore was also measured by the photo-cell.

Visual estimation

The intensity of the formaldehyde-induced fluorescence in the adrenergic cell bodies was estimated subjectively by assessing the fluorescence of the cells as 'negative', 'moderate' or 'intense' (Fig. 5). Cells with a very weak fluorescence were regarded as 'negative' as it was easy to confuse the specific fluorescence induced by formaldehyde and non-specific autofluorescence. The validity of such evaluation was tested by comparing the independant judgement of the two observers on the same 100 cells. All but four cells were judged to belong to the same classes by both observers (Table 1*a*). A further test was performed by comparing the subjective evaluation of the fluorescence intensity of 100 cells (negative, moderate or intense) with the fluorescence intensity of the same cells measured with the photometric method as described above. All but three of the examined 100 cells fell in the same classes (Table 1*b*). The photometric limits of

Table 1a

		First observer		
		negative	*moderate*	*intense*
Second observer	negative	31	1	
	moderate		31	3
	intense			34

Table 1b

		Visual estimation		
		negative	*moderate*	*intense*
Photometric estimation	negative	32	1	
	moderate	1	32	
	intense		2	31

the visual classes were: 'negative' = 0–8.3, 'moderate' = 8.3–16.8, and 'intense' = 16.8–35. The visual estimations appear, therefore, to follow the Weber-Fechner law, i.e. the sensation varies linearly with the logarithm of the stimulus.

The subjective evaluation thus showed a good reproducibility and the main intensity estimations were subsequently carried out by one observer only. The fluorescence of 1000 cells was visually estimated in each ganglion at a magnification of 250. The fluorescence intensity of all cells in three fields from the proximal, middle and distal part of each section was estimated. In every ganglion, 20–40 evenly spaced sections were thus examined so that the sampling included cells from all parts of the ganglia. Only the intensity of the cell body was taken into consideration.

When the fluorescence intensity was estimated visually, fewer cells were judged to exhibit an 'intense' fluorescence in ganglia with increased fluorescence (see Figs. 2 & 4), probably because of adaptation. Conversely, the photometric method tended to underestimate the number of 'negative' cells because light of other fluorescent structures behind and around them was included in the measurement. Thus, the two methods partially compensate for each other's deficiencies so that the real cell number in each category is likely to be between the values obtained with the two methods. In order to compare the values given by the two methods, the intensity values measured photometrically were ranked into the three corresponding classes estimated visually (Fig. 4). The intensity values measured photometrically as described above for the reliability

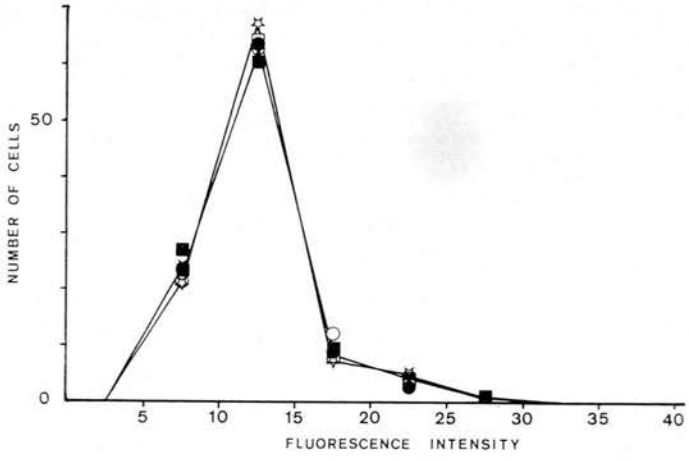


Figure 1. Plot in which four decentralized superior cervical ganglia are compared with a pair of control ganglia from an unoperated animal. The intensity of the fluorescence in the nerve cell bodies was measured photometrically. The number of cell bodies in classes of fluorescence intensity with 5 unit-wide limits is given by the symbols. Open stars and filled stars joined with lines: right and left ganglia, respectively, of an unoperated control animal. The mean values of the decentralized ganglia are indicated by the other signs which are not joined by lines to avoid further superposition of symbols. Open squares: decentralized ganglia of rats kept at 22°C for 5 days. Filled squares: decentralized ganglia of rats kept at 22°C for 16 days. Open circles: decentralized ganglia of rats kept at 5°C for 5 days. Filled circles: decentralized ganglia of rats kept at 5°C for 16 days. The values of decentralized ganglia represent means. The number of animals and the S.E.M.s are given in Fig. 3. Because of overlapping values, all the symbols are not visible, but this is not essential because the figure illustrates the similarity of the distributions.

test were taken as class limits for the three visual classes, 'negative', 'moderate' and 'intense'.

STATISTICAL ANALYSES

The numerical data were analysed statistically using an electronic computer (Sony 1 CC–2700 E) with programmed cards for the Student's *t*-test of matched pairs, the *t*-test for the difference of means and the χ^2 test. The *t*-test was applied to the corresponding columns of the intensity classes measured photometrically or visually. The mean percentage of the different intensity-classes were subjected to the χ^2 test.

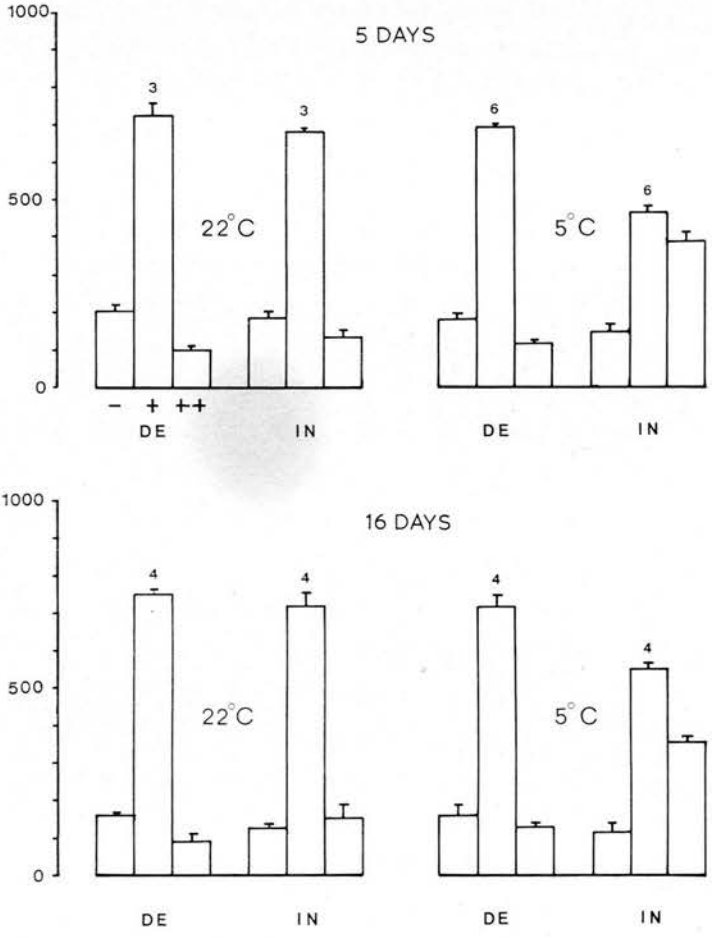


Figure 2. Visually estimated proportions of nerve cell bodies with negative (−), moderate (+) and intense (+ +) fluorescence in the superior cervical ganglion under experimental conditions. Each value represents the mean of estimations of a number of ganglia indicated by the small number above each column. Vertical bars represent the S.E.M. DE = denervated ganglia. IN = intact ganglia.

Results

General appearance of ganglia

In the ganglia dried under optimal conditions, the specific fluorescence in the cell bodies was present all through the nerve cell cytoplasm from the nuclear membrane to the periphery. In addition to the diffuse component, a particulate, intensely fluorescent component was present in the cytoplasm (Figs. 17 & 18), as has been described previously (Eränkö & Härkönen, 1963; Eränkö, 1972*a*). In many neurons the particulate component was present throughout the cytoplasm, while in others it was located peri-

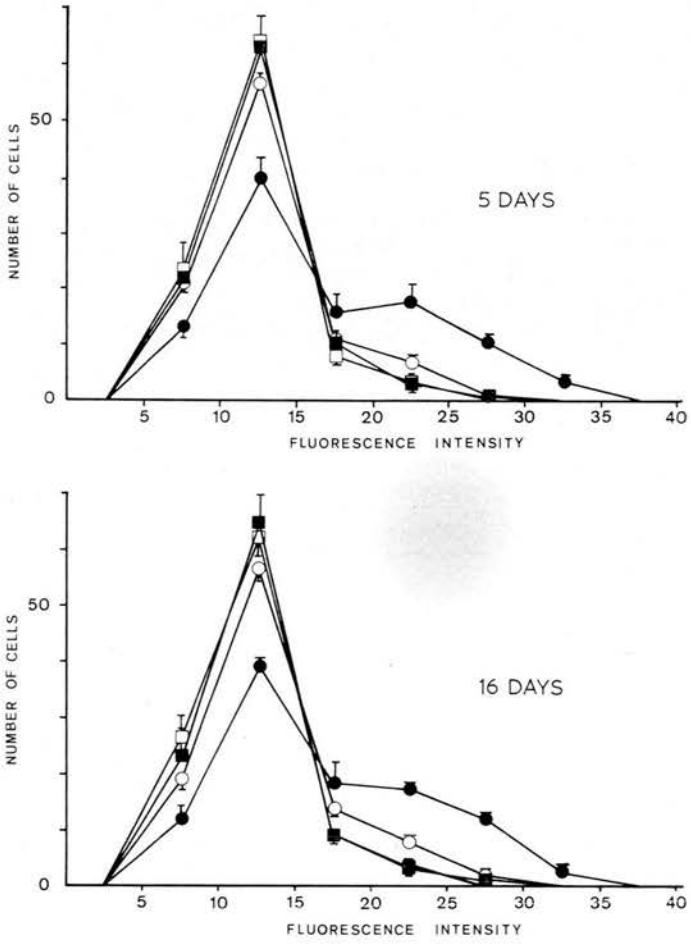


Figure 3. Frequency distribution of nerve cell bodies based on the intensity of fluorescence. The symbols represent the means. The vertical bars represent the S.E.M.s.

Upper diagram: 5-day-experiment. Open squares: denervated ganglia of rats (three) kept at room temperature (22°C). Open circles: intact ganglia of rats (three) kept at room temperature. Filled squares: denervated ganglia of rats (six) kept at 5°C. Filled circles: intact ganglia of rats (six) kept at 5°C.

Lower diagram: 16-day-experiment. Symbols as above. Each value represents the mean of four rats.

pherally or preferentially in the perinuclear region. The variability in the preservation of the subcellular catecholamine storage sites, even between nerve cells in the same ganglia, did not allow a reliable estimation of the relative changes of the two components in the different experiments.

Control ganglia

When right and left ganglia taken from unoperated control animal were compared, no statistically significant differences were observed (χ^2, $p > 0.05$). A comparison of the intensity of 100 cells from each ganglion is illustrated in Fig. 1 (continuous lines). The

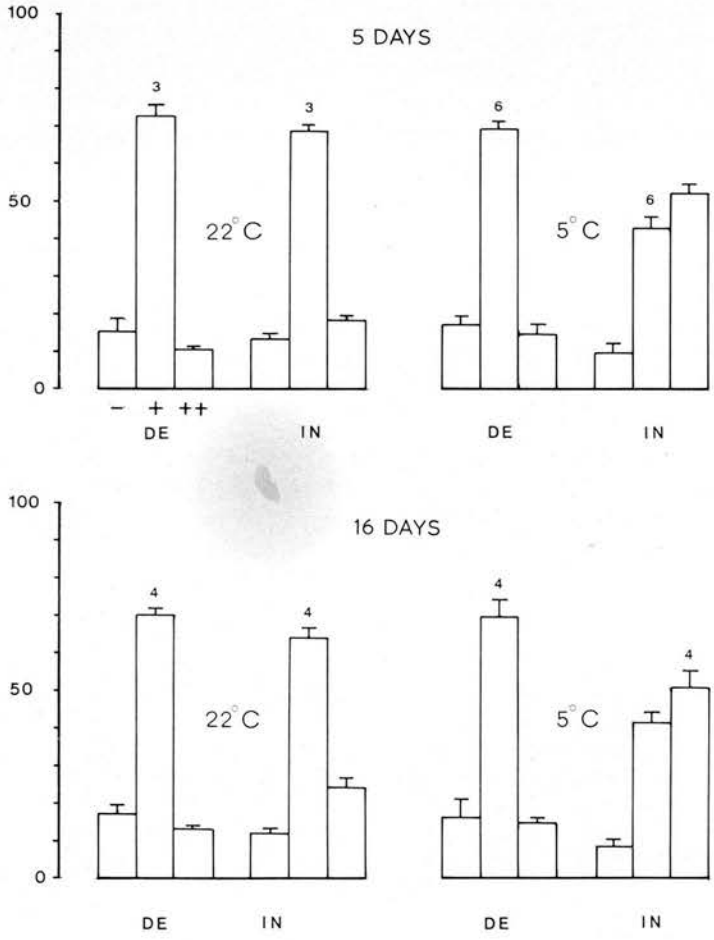


Figure 4. Diagrams showing the relative proportion of nerve cell bodies in different intensity classes as estimated photometrically. These data are to be compared with those in Fig. 2, in which the proportions were visually estimated. The proportions of nerve cell bodies with negative (−), moderate (+) and intense (+ +) fluorescence were measured photometrically as in Fig. 3 but intensity classes were formed to correspond to the visual classes. These values are based on 100 cells per ganglion. The number above each column indicates the number of ganglia, the vertical bars the S.E.M. DE = denervated ganglia. IN = intact ganglia.

relative proportions of the three types of cells were similar in both the photometric and the visual estimations. Of the cell bodies, 10–13% (values obtained by the two methods) showed 'intense' fluorescence, 15–18% showed no fluorescence and the rest, 69–73% showed a 'moderate' fluorescence. Non-fluorescent cells were usually larger and more numerous in the proximal pole of the ganglion, while the 'intense' cells were more numerous in the distal pole. These cells were generally of smaller size than the non-fluorescent cells. Fig. 5 shows a photomicrograph of a control ganglion.

Decentralized ganglia

The decentralized ganglia taken from animals kept at room temperature (22°C) did not differ significantly from the intact (unoperated) control ganglia either in the 5-day-experiment or in the 16-day-experiment ($p > 0.05$ in the χ^2 test in both cases) (Fig. 1). No difference was observed between decentralized ganglia at 5 and 16 days (Figs. 2 & 4, left histograms) by the χ^2 test or the t-test of differences of mean samples ($p > 0.05$ for all the methods of estimation). Comparison of the decentralized ganglia of animals exposed to cold (5°C) with decentralized ganglia of animals kept at 22°C for 5 and 16 days, showed no statistically significant differences (Figs. 1, 2 & 4) using the t-test of

---

Figures 5–8. Formaldehyde-induced amine fluorescence in the superior cervical ganglia of control and experimental animals. The exposure times and the developing times of the negatives and the prints are all identical. In all figures, bar = 50 μm.

Figure 5. Superior cervical ganglion of a control rat. The arrows indicate cell bodies with 'negative' fluorescence (short arrow), 'moderate' fluorescence (medium arrow) or 'intense' fluorescence (long arrow).

Figure 6. Intact superior cervical ganglion of a rat kept at room temperature for 5 days; the contralateral ganglion was decentralized. There is some increase in the intensity of the nerve cell fluorescence as compared with the control ganglion (Fig. 5).

Figure 7. Denervated ganglion of a rat kept at 5°C for 5 days. The cells show an essentially normal fluorescence range.

Figure 8. Intact contralateral ganglion of the rat exposed to cold shown in Fig. 7. Note the marked increase in the number of 'intense' cell bodies as compared with the decentralized ganglion in Fig. 7.

Figures 9–12. Formaldehyde-induced amine fluorescence in the superior cervical ganglia from 16-day-experiment rats. The exposure and developing times of the negatives and the prints were identical. In all figures, bar = 50 μm.

Figure 9. Decentralized ganglion of a rat kept at room temperature for 16 days.

Figure 10. Intact contralateral ganglion from the same rat. Note more numerous cell bodies with 'intense' fluorescence as compared with the decentralized side (Fig. 9).

Figure 11. Decentralized ganglion of a rat kept at 5°C for 16 days. The cells show a fluorescence range similar to that in Fig. 9.

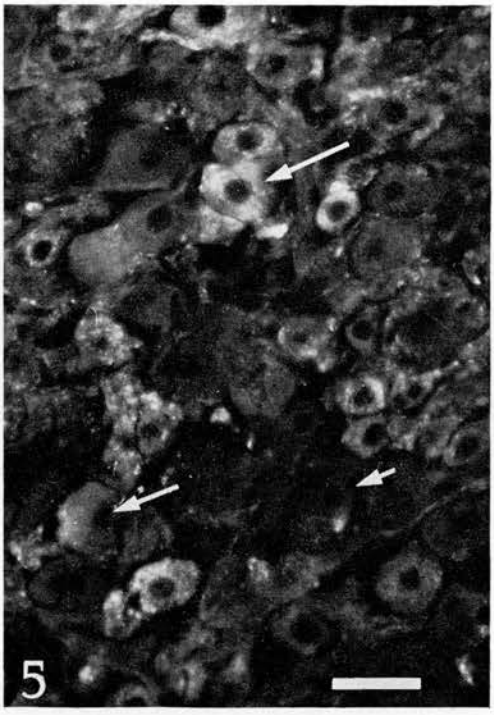
Figure 12. Intact contralateral ganglion of the same rat exposed to cold. There is a marked increase in the number of cell bodies with 'intense' fluorescence as compared with the decentralized side (Fig. 11).

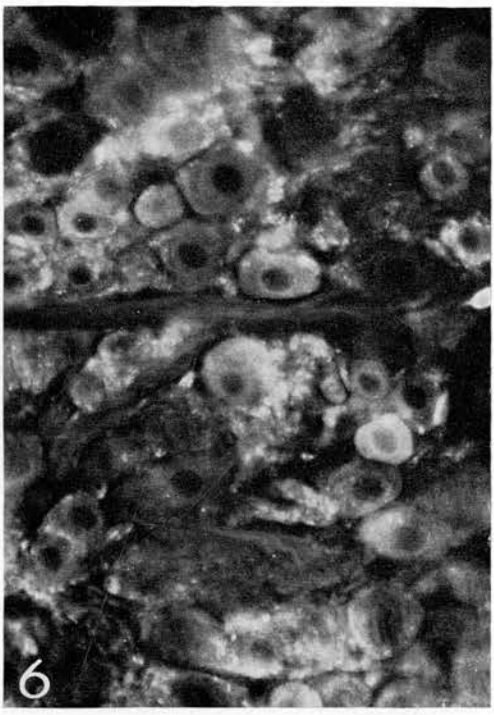
Figure 13. Decentralized ganglion of a rat kept at 5°C for 16 days. Bar = 50 μm.

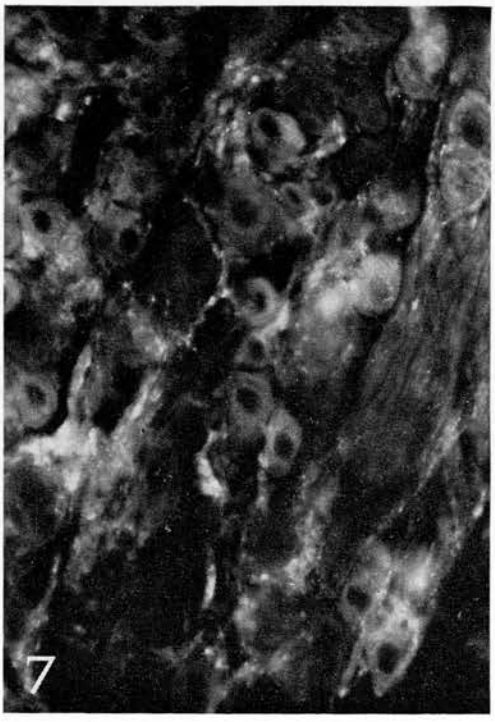
Figure 14. Intact ganglion of the rat shown in Fig. 13 photographed under identical conditions. The number of 'intense' cell bodies is strikingly increased. Magnification as in Fig. 13.

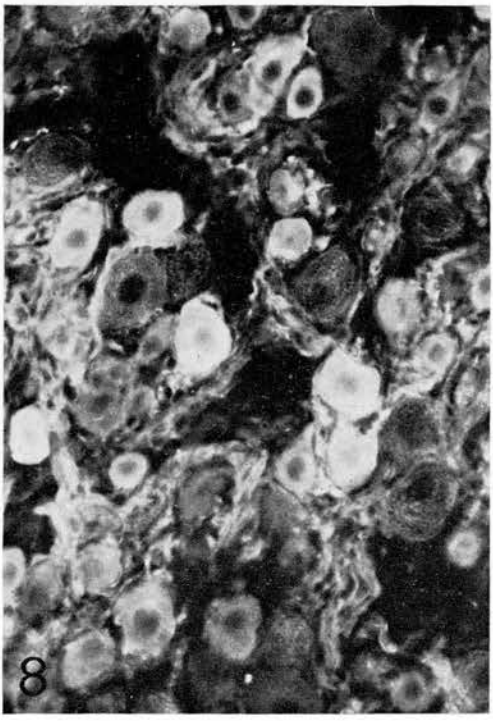
Figure 15. General view of a decentralized ganglion of a rat kept at 5°C for 5 days. Bar = 100 μm.

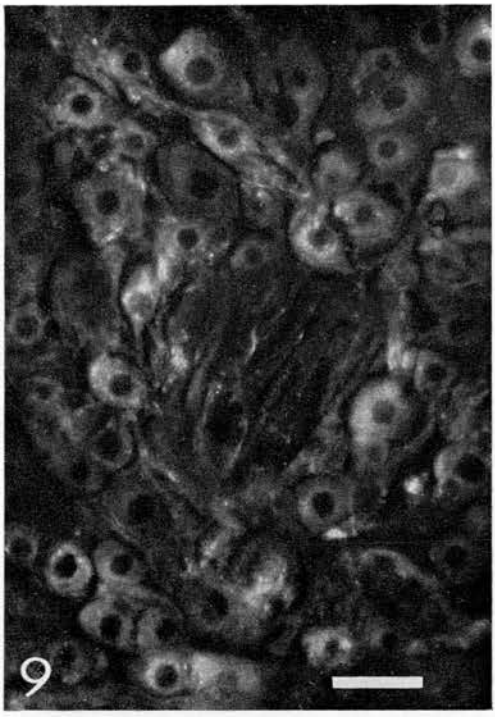
Figure 16. Photograph taken under identical conditions as Fig. 15 of the contralateral ganglion. The larger number of 'intense' cell bodies distributed everywhere gives a patchy appearance to the ganglion. Magnification as in Fig. 15.

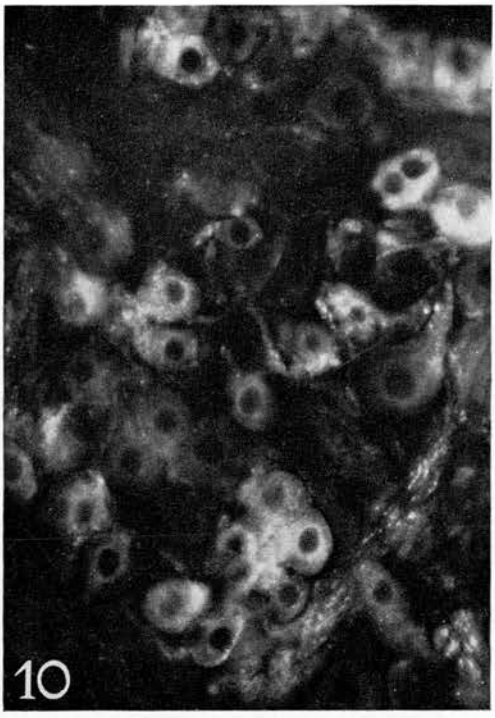

5

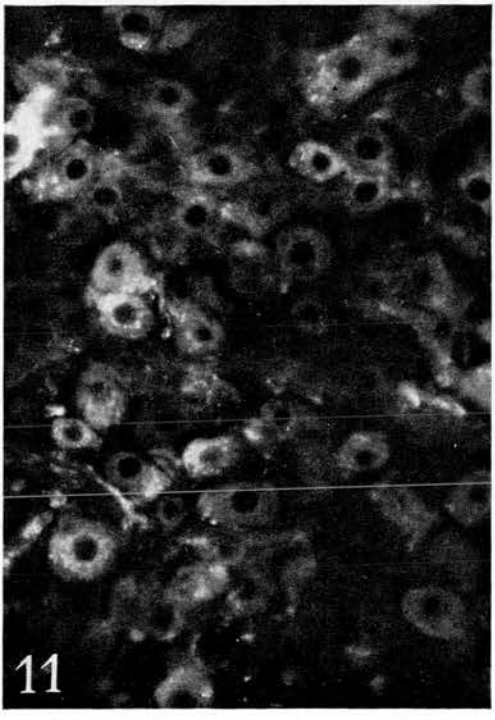

6

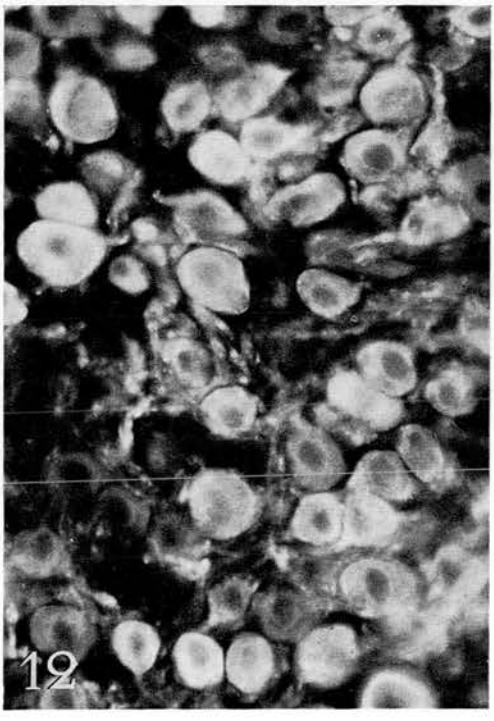

7

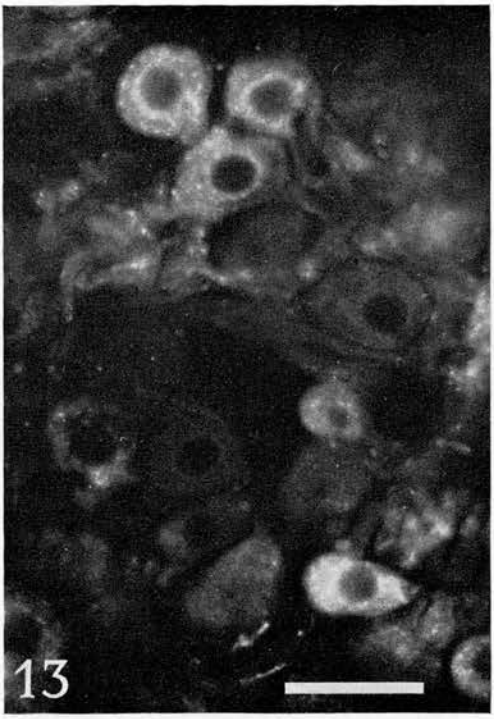

8

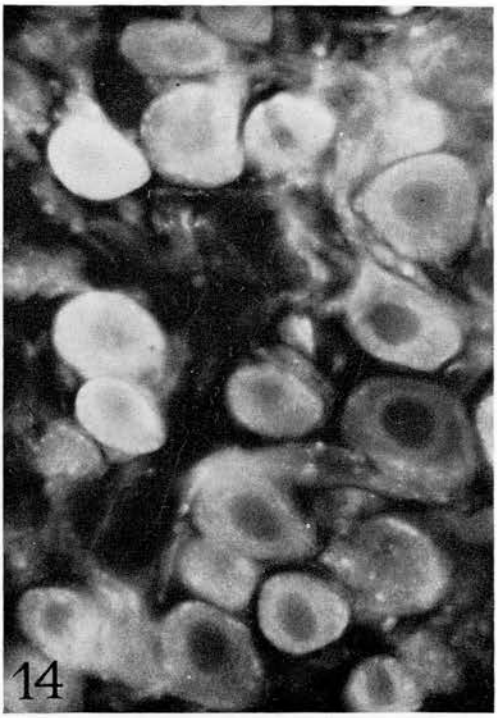

9

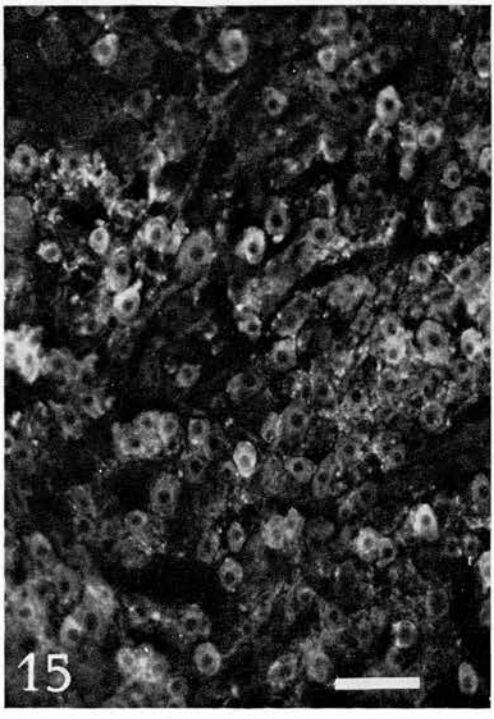

10

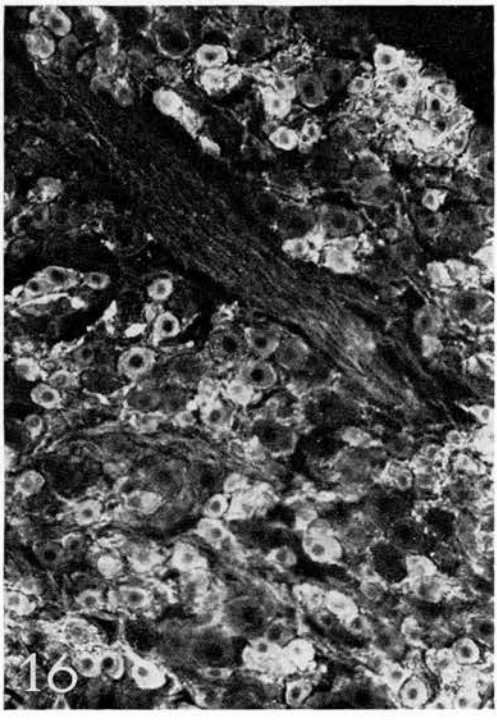

11

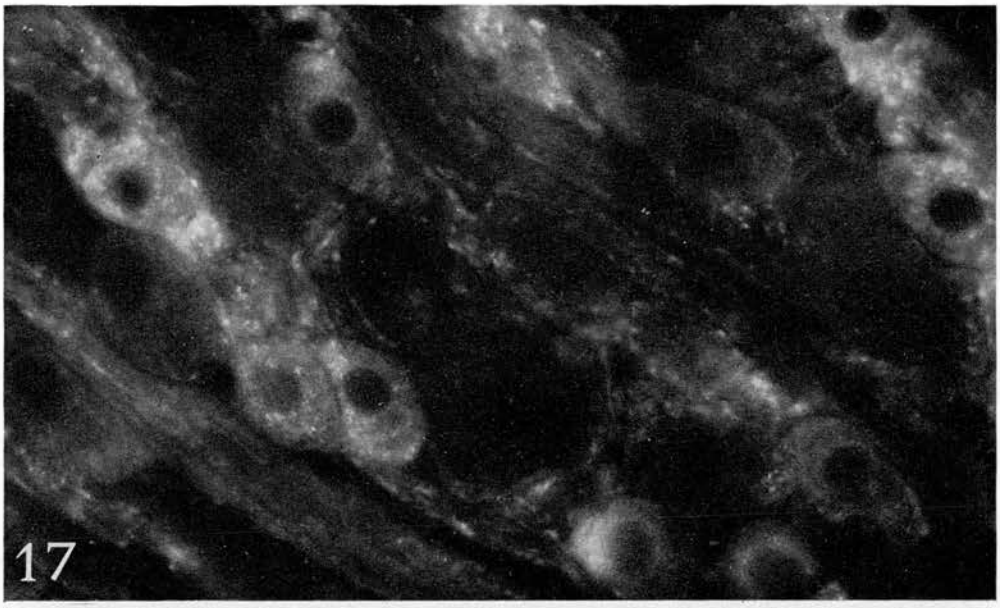

12


13


14


15


16



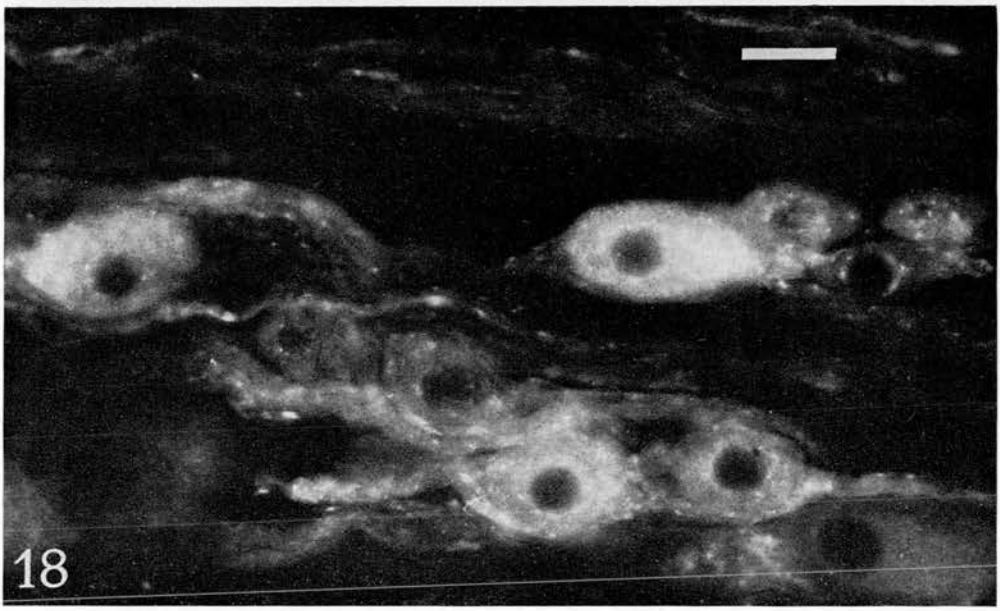


Figure 17. High magnification of a decentralized ganglion of a rat kept at 5°C for 16 days. Bar = 25 μm.

Figure 18. Intact ganglion of the same rat. The photograph was taken, developed and printed in the same way as that shown in Fig. 17. Evenly distributed and particulate components of fluorescence are clearly visible in the cytoplasm of the nerve cells. Magnification as in Fig. 17.

differences of samples means ($p > 0.05$), thus suggesting that the exposure to cold had no effect on the decentralized sympathetic ganglia (see Figs. 5 & 7, 9 & 11).

Intact ganglia

Animals kept at room temperature. The intact and decentralized ganglia in the same animals kept at room temperature (Figs. 2–4) were compared by the matched pairs *t*-test. In the 15-day-experiment, a slight increase of the fluorescence intensity in some cell bodies was observed in the intact ganglion (Fig. 6) as compared with the denervated ganglion (Fig. 5). This slight difference is also apparent in the photometric measurements (Fig. 3, top). However, the difference was not statistically significant ($p > 0.05$ both in the photometric and visual estimations).

Some cells also showed an increased fluorescence in the intact ganglia in the 16-day-experiment (Figs. 9 & 10). In the photometric measurement (Fig. 3), the matched pairs *t*-test showed a highly significant ($p < 0.005$) difference, and in both visual (Fig. 2) estimations and photometric estimations translated into visual ones (Fig. 4) there was a statistically significant difference ($p < 0.025$ and < 0.05, respectively) between the columns of the 'intense' cells, whose proportion increased from 9–15% in the denervated to 15–24% in the intact ganglion. (Figs. 6 and 10 show photomicrographs of intact ganglia after 5 and 16 experiment-days.)

Animals kept at 5°C. The effect of cold on the intact ganglia was studied by comparing, with the matched pairs *t*-test, the intact ganglia of rats exposed to 5°C for 5 or 16 days with the contralateral denervated ganglia.

After 5 days, a marked increase in the number of 'intense' cells was observed in the intact ganglia with all the methods of fluorescence estimation, as compared with the decentralized ganglia. By visual estimation (Fig. 2), the proportion of 'intense' cells increased from 12% in the denervated to 39% in the intact ganglia ($p < 0.001$). The relative number of 'moderate' cells decreased from 69% in the decentralized ganglia to 47% in the intact ones ($p < 0.001$). The mean relative number of 'negative' cells decreased from 18 to 15% but the difference was not statistically significant ($p > 0.05$). In the photometric estimation translated to the visual classes (Fig. 4), the cold-induced increase in the relative number of 'intense' cells was even more marked, from 14% in the denervated ganglia to 52% in the intact ones ($p < 0.001$). There was also a decrease in the relative number of the 'moderate' cells from 69 to 43% ($p < 0.005$). The mean decrease of the population of the 'negative' cells from 17 to 9% was not highly significant ($p < 0.05$). Fig. 3 (top) shows clearly the changes of the intensity measured photometrically of the cells in the intact superior cervical ganglion in rats exposed to cold for 5 days. Fig. 8 shows a photomicrograph of an intact ganglion with a large number of intense cells as compared with the decentralized contralateral ganglion (Fig. 7).

The cold-induced 'intense' cells in the intact ganglia were increased in all parts of the ganglia. The increase in 'intense' cells produced a patchy appearance of the ganglia (Fig. 15), as compared to the decentralized ganglia, which were more homogeneous (Fig. 16).

An increased number of 'intense' cell bodies was also observed in the intact ganglia after 16 days of exposure to cold (Figs. 2–4, bottom). By visual estimation (Fig. 2, bottom), the proportion of 'intense' cells increased from 13% in the denervated ganglia

to 35% in the intact ones ($p < 0.001$) and the amount of the 'moderate' cells decreased from 71 to 55% ($p < 0.005$). The slight decrease in the number of the 'negative' cells from 16% in the decentralized ganglia to 11% in the intact ones, was not statistically significant ($p > 0.05$). By photometric estimations expressed as visual classes (Fig. 4, bottom), the differences were again more marked. The proportion of 'intense' cells increased from 15% in the decentralized ganglia to 51% in the intact ganglia ($p < 0.005$), while the relative number of 'moderate' cells decreased from 70 to 41% ($p < 0.005$). The decrease in this case, from 16 to 8% in the mean percentage of the 'negative' cells, was also not statistically significant ($p > 0.05$). Fig. 3 (top) shows the changes in the photometric measurements of the cells in the intact superior cervical ganglia of rats exposed to cold for 16 days. The marked increase in the 'intense' cells in the intact ganglion, as compared with the contralateral decentralized one, is apparent in the microphotographs (Figs. 11 & 12, 13 & 14, 17 & 18). These microphotographs show a region containing particularly numerous intensely fluorescent cell bodies.

The increase in the intensity of the cell bodies in the intact ganglia of rats exposed to cold for 16 days was similar to the increase found after 5 days of cold exposure. The χ^2 test between the sets of data obtained with all the methods employed failed to show statistically significant differences ($p > 0.05$) between the 5- and 16-day experiments.

When the intact ganglia were compared in animals exposed to cold and animals kept at room temperature, a marked increase in the relative number of 'intense' cells occurred both after 5 and 16 days. The χ^2 test showed a statistically highly significant difference ($p < 0.005$). The *t*-test for difference of sample means showed highly significant differences ($p < 0.005$) in the 'moderate' and 'intense' cells in both visual and photometric estimation (see Figs. 2–4) while the differences in the 'negative' cells were not statistically significant ($p > 0.05$) with either estimation method.

Discussion

This study shows that the fluorescence histochemical technique for the localization of catecholamines can be used to detect changes in the fluorescence of the adrenergic cell bodies in experimental conditions. Since the changes and their magnitude were detected by both visual and photometric methods, the histochemical technique represents a useful tool to investigate morphological correlates of functional events in sympathetic ganglia.

This study shows that exposure of rats to cold results in a marked increase in the intensity of the formaldehyde-induced fluorescence of some adrenergic cell bodies. It could be considered that cold might have a direct effect on the adrenergic neurons or that it might activate some circulating factor responsible for some part of the increased fluorescence. This is unlikely since no differences were seen in decentralized ganglia of rats exposed to cold, as compared to decentralized ganglia of rats kept at room temperature.

The increase in the fluorescence intensity, which is indicative of an increase in the noradrenaline content of the cell bodies (Ritzen, 1967), did not occur in the decentralized contralateral ganglia. This shows that the fluorescence increase was mediated by the preganglionic nerve fibres.

Exposure to cold results in an increase of the urinary excretion of noradrenaline, which originates mainly from sympathetic nervous tissue whose activity is increased (Leduc,

1961; Johnson, 1964, 1966; Pouliot, 1966; Motelica, 1969). Since the noradrenaline content of tissues remains unchanged under such conditions (Leduc, 1961) it has been suggested that an increased synthesis of noradrenaline occurs as a result of increased neuronal activity (Pouliot, 1966) and this has been confirmed by Gordon *et al.* (1966) and Dairman & Udenfriend (1970). Exposure to cold for 1–4 days has been shown to induce an increased synthesis of tyrosine hydroxylase and dopamine hydroxylase in the superior cervical ganglia of the rat, an effect which is mediated by pre-ganglionic nerve fibres (Thoenen, 1970). The increase in the fluorescence of some adrenergic cell bodies shown in the present work is a result of an increased production/consumption ratio of noradrenaline. Since no changes in noradrenaline content of sympathetic ganglia were detected after a short pre-ganglionic stimulation (Reinert, 1963; Weiner & Mosimann, 1970; Bhatnagar & Moore, 1971), the increased fluorescence observed in this work is unlikely to be due to the rapid increase of noradrenaline synthesis which is triggered by a reduction of the transmitter levels due to its release (Weiner *et al.*, 1972). Therefore, the increase in the fluorescence of some adrenergic cell bodies shown in the present work probably reflects the increase in the synthetic enzymes as a result of a prolonged increase in pre-ganglionic activity, representing a correlate of the 'trans-synaptic induction'. The accumulation of noradrenaline in the adrenergic cell bodies which accompanies the induction of tyrosine and dopamine hydroxylases, also indicates that the rate of disappearance of noradrenaline from the cell bodies by either metabolic degradation, axonal transport to the nerve terminals or release, is smaller than the rate of synthesis. It is interesting that the enzyme induction produced by the nerve growth factor (Thoenen *et al.*, 1971*a*) is also accompanied by an accumulation of noradrenaline in the cell bodies (Olson, 1967).

The cold-induced increase in the noradrenaline content of the adrenergic cell bodies was, in this study, maintained for up to 16 days. The previous observation that the increase in noradrenaline excretion is maintained throughout the cold-exposure period for at least 15 days (Leduc, 1961; Pouliot, 1966), supports the idea that long-lasting reflex stimulation of the sympathetic system results in a prolonged increase of noradrenaline synthesis and storage in the cell body, as well as continuously increased noradrenaline release from the terminals, all of which last for the whole period of increased pre-ganglionic activity with no adaptation of the adrenergic neuron. Further investigations are required to establish whether an even longer period of increased pre-ganglionic activity would result in steadily higher enzyme levels and noradrenaline storage. It would also be of interest to study how soon the return to normal pre-ganglionic activity is accompanied by restoration of the original enzyme and noradrenaline levels.

As the half-life of dopamine hydroxylase in sympathetic ganglia is about 12 to 15 hr (Thoenen *et al.*, 1971*b*; Axelrod, 1972) and that of tyrosine hydroxylase is about 8 days (Mueller *et al.*, 1969*b*), a return to normal levels is likely to occur in a relatively short time. On this basis and assuming that no adaptation occurs, the level of intensity of fluorescence of the adrenergic cell bodies may reflect, with some delay, the level of activity of the pre-ganglionic fibres synapsing on them, although other factors, such as nerve growth factor and hormones, may be also involved. Not all the cells showed an increased fluorescence intensity; this suggests that only part of the adrenergic neurons of the superior cervical ganglion are affected by the increase in activity of the pre-ganglionic

fibres induced by cold. Mainly those directly or indirectly involved in the thermoregulation can be expected to be affected. Exposure to cold, for instance, increases the noradrenaline turnover in the heart but not that in the salivary glands (Costa *et al.*, 1969). Little is known about the relation between frequency of firing of pre-ganglionic nerve fibres and induction of enzymes. There might be a threshold firing frequency or specific spatial and temporal patterns of pre-ganglionic activity which trigger the trans-synaptic mechanism of enzyme induction. Since about 20–30% of the cells estimated visually and photometrically showed an increased fluorescence induced by cold, these are probably the proportion of adrenergic neurons in the superior cervical ganglion which receive an increased pre-ganglionic activity in a cold environment. The calculation has to be interpreted with great care because the percentages given represent changes between two classes of fluorescence intensity, while changes within a class would not have been detected.

The cells which showed an increase in the fluorescence intensity are mostly 'intense' cells shifted from the column of the 'moderate' cells, while little contribution is made by the 'negative' cells. The small reduction of the mean percentage of the 'negative' cells may represent a shift of 'weakly' fluorescent adrenergic neurons into the 'moderate' class, while the rest might represent cholinergic cell bodies. In sympathetic ganglia, a small percentage of neurons are considered cholinergic on the basis of their lack of fluorescence, a marked acetylcholinesterase activity and the presence of choline acetyltransferase activity (Hamberger & Norberg, 1963; Hamberger *et al.*, 1963; Bell & McLean, 1967; Robinson & Mann, 1971; Yamauchi & Lever, 1971). In the rat, non-fluorescent neurons appear to be associated with a high non-specific cholinesterase rather than with acetyl-cholinesterase activity (Eränkö, 1964, 1972*b*; Härkönen, 1964) and no inverse correlation was found between fluorescence intensity and acetylcholinesterase activity in the nerve cell bodies (Eränkö & Härkönen, 1964; Härkönen, 1964; Perry, 1971).

The lack of effect of pre-ganglionic nerve division on the intensity of fluorescence in the adrenergic cell bodies, as shown in this work, suggests that decentralization causes no reduction in the amount of the enzymes of noradrenaline synthesis. It is possible that the reduction of physiological pre-ganglionic activity caused by decentralization was not sufficient to involve changes in the level of tyrosine and dopamine hydroxylases. The low frequency of the firing of the tonically active pre-ganglionic fibres (Polosa, 1968; Folkow & Neil, 1971) which corresponds to a similar low frequency of firing of the adrenergic neurons in the superior cervical ganglia (Mirgorodsky & Skok, 1969), and the surprisingly low proportion of cells tonically active in the superior cervical ganglion (Erulkar & Woodward, 1968; Chalazonitis & Gonella, 1971) support this view. No change in dopamine hydroxylase has been found 9 days after decentralization of the superior cervical ganglion of the rat (Axelrod, 1972) and no significant changes in tyrosine hydroxylase after 2–7 days (Sedvall & Kopin, 1967; Thoenen *et al.*, 1969*b*). However, a small transient increase in noradrenaline content from 14 to 21 days has been found in the decentralized superior cervical ganglion of the cat (Kirpekar *et al.*, 1962; Fischer & Snyder, 1965). It is also possible that small changes were not detected by our methods.

Denervation of a ganglion resulted in this study in a small increase in the proportion of the 'intense' cell bodies in the controlateral, intact ganglion. This finding suggests that

there is a compensatory increase of the resting tonic sympathetic activity in the intact side following decentralization of the contralateral side.

Acknowledgements

The authors are greatly indebted to Dr Daniela Costa for the assistance in all stages of this work, which was supported by the Sigrid Jusélius Foundation.

References

AXELROD, J. (1972). Dopamine-β-hydroxylase: Regulation of its synthesis and release from nerve terminals. *Pharmac. Rev.* **24,** 233–43.

BELL, C. & MCLEAN, J. R. (1967). Localization of norepinephrine and acetylcholinesterase in separate neurons supplying the guinea-pig vas deferens. *J. Pharmac. exp. Ther.* **157,** 69–73.

BHATNAGAR, R. K. & MOORE, K. E. (1971). Effect of electrical stimulation, α-*m*-tyrosine and desmethylimipramine on the norepinephrine content of neuronal cell bodies and terminals. *J. Pharmac. exp. Ther.* **178,** 450–63.

CHALAZONITIS, A. & GONELLA, J. (1971). Activité electrique spontanée des neurones du ganglion stellaire du chat. Etude par microélectrode extracellulaire. *J. Physiol. (Paris)* **63,** 599–609.

COSTA, E., NEFF, N. H. & NGAI, S. H. (1969). Regulation of metaraminol efflux from rat heart and salivary gland. *Br. J. Pharmac.* **36,** 153–60.

DAIRMAN, W. & UDENFRIEND, S. (1970). Effect of ganglion blocking agents on the increased synthesis of catecholamines resulting from α-adrenergic blockade or exposure to cold. *Biochem. Pharmac.* **19,** 979–84.

ERÄNKÖ, O. (1964). Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. *J. Histochem. Cytochem.* **12,** 487–9.

ERÄNKÖ, O. (1967). The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. *J. R. microsc. Soc.* **87,** 259–76.

ERÄNKÖ, O. (1972*a*). Light and electron microscopic histochemical evidence of granular and non-granular storage of catecholamines in the sympathetic ganglion of the rat. *Histochem. J.* **4,** 213–24.

ERÄNKÖ, L. (1972*b*). Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. *Brain Res.* **46,** 159–75.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1963). Histochemical demonstration of fluorogenic amines in the cytoplasm of sympathetic ganglion cells of the rat. *Acta physiol. scand.* **58,** 285–6.

ERÄNKÖ, O. & HÄRKÖNEN, M. (1964). Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. *Acta physiol. scand.* **61,** 299–300.

ERULKAR, S. D. & WOODWARD, J. K. (1968). Intracellular recording from mammalian superior cervical ganglion *in situ*. *J. Physiol. (Lond.)* **199,** 189–203.

FISCHER, J. E. & SNYDER, S. (1965). Disposition of norepinephrine-H^3 in sympathetic ganglia. *J. Pharmac. exp. Ther.* **150,** 190–5.

FOLKOW, B. & NEIL, E. (1971). *Circulation.* New York, London, Toronto: Oxford University Press.

GORDON, R., SPECTOR, S., SJOERDSMA, A. & UDENFRIEND, S. (1966). Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold. *J. Pharmac. exp. Ther.* **153,** 440–7.

HAMBERGER, B. & NORBERG, K-A. (1963). Monoamines in sympathetic ganglia studied with fluorescence microscopy. *Experientia* **19,** 580–91.

HAMBERGER, B., NORBERG, K-A. & SJÖQVIST, F. (1963). Cellular localization of monoamines in sympathetic ganglion of the cat. A preliminary report. *Life Sci.* **9,** 659–61.

HÄRKÖNEN, M. (1964). Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and postganglionic nerve division. *Acta physiol. scand.* **63,** *Suppl.* 237, 1–94.

JOHNSON, G. E. (1964). The influence of chlorpromazine on the catecholamine excretion of normal and cold acclimated rats. *Acta physiol. scand.* **60,** 129–33.

JOHNSON, G. E. (1966). The effect of cold exposure on the catecholamine excretion of rats treated with decaborane. *Acta physiol. scand.* **68,** 129–33.

KIRPEKAR, S. M., CERVONI, P. & FURCHGOTT, R. F. (1962). Catecholamine content of the cat nictitating membrane following procedures sensitizing it to norepinephrine. *J. Pharmac. exp. Ther.* **135,** 180–90.

LEDUC, J. (1961). Catecholamine production and realease in exposure and acclimation to cold. *Acta physiol. scand.* **53,** *Suppl.* 183.

MIRGORODSKY, V. N. & SKOK, V. I. (1969). Intracellular potentials recorded from a tonically active mammalian sympathetic ganglion. *Brain Res.* **15,** 570–2.

MOLINOFF, P. B., BRIMIJOIN, W. S., WEINSHILBAUM, R. M. & AXELROD, J. (1970). Neurally mediated increase of dopamine-β-hydroxylase activity. *Proc. natn. Acad. Sci. U.S.A.* **66,** 453–8.

MOTELICA, I. (1969). Urinary excretion of catecholamines and vanilmandelic acid in rats exposed to cold. *Acta physiol. scand.* **76,** 393–5.

MUELLER, R. A., THOENEN, H. & AXELROD, J. (1969*a*). Increase in tyrosine hydroxylase activity after reserpine administration. *J. Pharmac. exp. Ther.* **169,** 74–9.

MUELLER, R. A., THOENEN, H. & AXELROD, J. (1969*b*). Inhibition of trans-synaptically increased tyrosin hydroxylase activity by cycloheximide and actinomycin D. *Mol. Pharmacol.* **5,** 463–9.

OLSON, L. (1967). Outgrowth of sympathetic adrenergic neurons in mice treated with a nerve-growth factor (NGF). *Z. Zellforsch. mikrosk. Anat.* **81,** 155–73.

PERRY, R. (1971). A histochemical study of cholinesterases in sympathetic ganglia. *J. Anat.* **110,** 499.

PLOEM, J. S. (1967). The use of vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z. wiss. Mikrosk,* **68,** 129–42.

POLOSA, L. (1968). Spontaneous activity of sympathetic pre-ganglionic neurons. *Can. J. Physiol. Pharmac.* **46,** 887–96.

POULIOT, M. (1966). Catecholamine excretion in adreno-demedullated rats exposed to cold after chronic guanethidine treatment. *Acta physiol. scand.* **68,** 164–8.

REINERT, H. (1963). Role and origin of noradrenaline in the superior cervical ganglion. *J. Physiol. (Lond.)* **167,** 18–29.

RITZÉN, M. (1967). Cytochemical identification and quantitation of biogenic monoamines. *M.D. Thesis, Stockholm.*

ROBINSON, P. M. & MANN, S. P. (1971). Histochemical localization of choline acetyltransferase in an autonomic ganglia. *J. Anat.* **110,** 500.

SEDVALL, G. C. & KOPIN, I. J. (1967). Influence of sympathetic denervation and nerve impulse activity on tyrosine hydroxylase in the rat submaxillary gland. *Biochem. Pharmoc.* **16,** 39–46.

THOENEN, H. (1970). Induction of tyrosine hydroxylase in peripheral and central adrenergic neurons by cold exposure of rats. *Nature (Lond.)* **228,** 861–2.

THOENEN, H. (1972). Comparison between the effect of neuronal activity and nerve growth factor on the enzymes involved in the synthesis of norepinephrine. *Pharmac. Rev.* **24,** 255–67.

THOENEN, H., ANGELETTI, P. U., LEVI-MONTALCINI, R. & KETTLER, R. (1971*a*). Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine-β-hydroxylase in the rat superior cervical ganglia. *Proc. natn. Acad. Sci. U.S.A.* **68,** 1598–602.

THOENEN, H., KETTLER, R., BURKARD, W. & SANER, A. (1971*b*). Neurally mediated control of enzymes involved in the synthesis of norepinephrine, are they regulated as an operational unit? *Naunyn Schneid. Arch. exp. Path. Pharm.* **270,** 146–60.

THOENEN, H., MUELLER, R. A. & AXELROD, J. (1969*a*). Trans-synaptic induction of tyrosine hydroxylase. *J. Pharmac. exp. Ther.* **169,** 249–54.

THOENEN, H., MUELLER, R. A. & AXELROD, J. (1969*b*). Increased tyrosine hydroxylase activity after drug-induced alteration of sympathetic transmission. *Nature (Lond.)* **221,** 1264.

WEINER, N., CLOUTIER, G., BJUR, R. & PFEFFER, R. I. (1972). Modification of norepinephrine synthesis in intact tissue by drugs and during short term adrenergic nerve stimulation. *Pharmac. Rev.* **24,** 203–21.

WEINER, N. & MOSIMANN, W. F. (1970). The effect of insulin on the catecholamine content and tyrosine hydroxylase activity of cat adrenal glands. *Biochem. Pharmac.* **19**, 1189–99.

YAMAUCHI, A. & LEVER, J. D. (1971). Correlations between formol fluorescence and acetylcholinesterase (AchE) staining in the superior cervical ganglion of normal rat, pig and sheep. *J. Anat.* **110**, 435–43.

Brain Research, 67 (1974) 457–466

HYDROCORTISONE-INDUCED INCREASE IN THE HISTOCHEMICALLY DEMONSTRABLE CATECHOLAMINE CONTENT OF SYMPATHETIC NEURONS OF THE NEWBORN RAT

MARCELLO COSTA*, OLAVI ERÄNKÖ AND LIISA ERÄNKÖ

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki SF 00170 (Finland)

(Accepted August 28th, 1973)

SUMMARY

Newborn rats were subcutaneously injected with 20 mg/kg body weight of hydrocortisone acetate each day for 5 days and were killed together with untreated controls 5 h or 10 days after the last injection. Histochemically demonstrable formaldehyde-induced fluorescence was studied in freeze-dried superior cervical ganglia and in stretch preparations of the intestine and the iris. Fluorescence intensity was measured by visual estimation and microscope photometry.

Hydrocortisone treatment caused a statistically significant increase in the number of intensely fluorescent nerve cell bodies and a corresponding decrease in the number of moderately fluorescent nerve cell bodies in the superior cervical ganglia. In the Auerbach's plexus of hydrocortisone-treated rats all fluorescent fibers were more clearly delineated, showed more often varicosities and exhibited a more intense fluorescence, as compared with those of the control rats. The network of adrenergic nerve fibers in the iris showed, likewise, significant increase in the fluorescence after treatment with hydrocortisone. The increased nerve fiber fluorescence was observed also 10 days after the last hydrocortisone injection.

It is concluded that hydrocortisone causes an increase in the content of noradrenaline in the sympathetic neurons, probably by increased synthesis.

INTRODUCTION

Glucocorticoids have no appreciable effect on extra-adrenal chromaffin tissue[7]

* Sigrid Jusélius Foundation Research Fellow in the Department of Anatomy, University of Helsinki, April–July 1973. Present address: Department of Zoology, University of Melbourne, Parkville 3052, Victoria, Australia.

or the adrenergic nerves[14] of adult rats. However, in newborn rats, hydrocortisone has dramatic effects on the extra-adrenal chromaffin tissue, causing hyperplasia[7,11], the appearance of adrenaline[8,18] and the induction of phenylethanol-N-methyltransferase[2,19]. Hydrocortisone has also been reported to cause increases in the protein synthesis and in the sizes of the nerve cell bodies in the superior cervical ganglia of newborn rats[10].

In the present study, histochemical evidence is presented for a hydrocortisone-induced increase in the catecholamine content of the sympathetic adrenergic neurons of the newborn rat.

MATERIALS AND METHODS

Experimental

Six litters of 6–10 rats, descendants of Sprague–Dawley strain, were used. Three litters were injected subcutaneously with 20 mg/kg body weight of hydrocortisone acetate (Organon) each day for 5 days. Two litters of injected rats were killed 5 h after the last injection, and one litter 10 days after the last injection. The non-injected rats of litters of the same age served as controls. Instead of littermate controls, rats born on the same day in separate litters were used, because with the former procedure the mothers tended to favor the untreated control rats over their injected littermates[7].

Fluorescence histochemistry

The rats were killed by cutting the spinal cord and the aorta at the level of the heart. The superior cervical ganglia were quickly removed and frozen in isopentane precooled with liquid nitrogen and were subsequently dried at −45 °C for 7 days[4]. They were then exposed to formaldehyde vapor generated from paraformaldehyde powder equilibrated with air of 60% relative humidity. After exposure to formaldehyde for 30 min at 50 °C and then for 1 h at 80 °C the ganglia were embedded in Epon. Serial sections of 5 μm were cut with a glass knife[6].

The iris and muscular layers of the small intestine with the Auerbach plexus were stretched on slides and air-dried in a desiccator over phosphorus pentoxide for 1 h. The slides were exposed to formaldehyde vapor as indicated above. The sections and the stretched preparations were mounted in Entellan (E. Merck, Darmstadt) and examined with an Ortholux microscope (E. Leitz, Wetzlar) fitted with an epiilluminator[16], the filters 3 mm BG 38, 3 mm BG 3, TAL 406 and K 470, and the Osram HBO 200 lamp.

Estimation of fluorescence intensity

The fluorescence intensities of the developing nerve cell bodies of the superior cervical ganglia were estimated visually as 'negative', 'moderate' or 'intense' (for reliability and details of the method see ref. 3). Two hundred cells in 10–15 visual fields from all parts of the ganglia were examined at ×400 magnification. Both visual and photometric estimations were applied to the stretched iris and intestinal preparations. Photometric estimations of the fluorescence intensities of the adrenergic nerve

fibers were made with the Ortholux automatic camera. The intensity of fluorescence was measured by recording the time of exposure with a standard setting of the camera. The fluorescence intensity is inversely proportional to the exposure time. Altogether 5–10 fields were estimated in each stretch preparation at $\times 250$ magnification.

Statistical analysis

Statistical analysis was performed by applying Student's t-test for the difference of sample means.

RESULTS

Superior cervical ganglion

Cell bodies with fluorescence of varying intensity were observed in the control ganglia, as has been described previously[5]. Fluorescence intensity estimations were performed only in the 5-day experiment (Fig. 1). The relative number of cells in the 'intense' class (++) was approximately tripled ($P < 0.001$) in the hydrocortisone-treated group. The class of cells with a moderate fluorescence (+) was reduced by about one-third ($P < 0.001$), while there was no significant change in the negative (−) class ($P > 0.05$).

It was easily possible to distinguish between the small intensely fluorescent (SIF) cells[6] and the adrenergic nerve cell bodies in the ganglia of both control and treated rats. In the present study, no attention was paid to the changes in the small intensely fluorescent cells which are the subject of a previous report[7]. Figs. 2 and 3 illustrate the large number of ganglion cells with an increased fluorescence intensity in the ganglia of the hydrocortisone-treated rats, as compared with those of the controls.

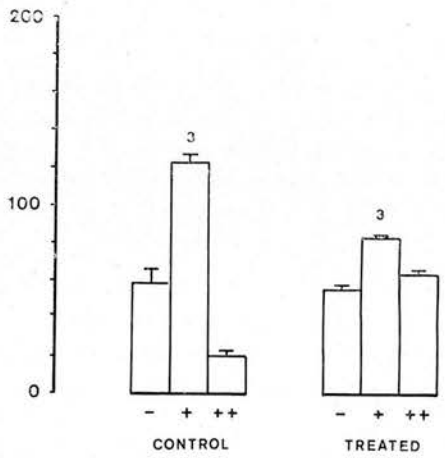
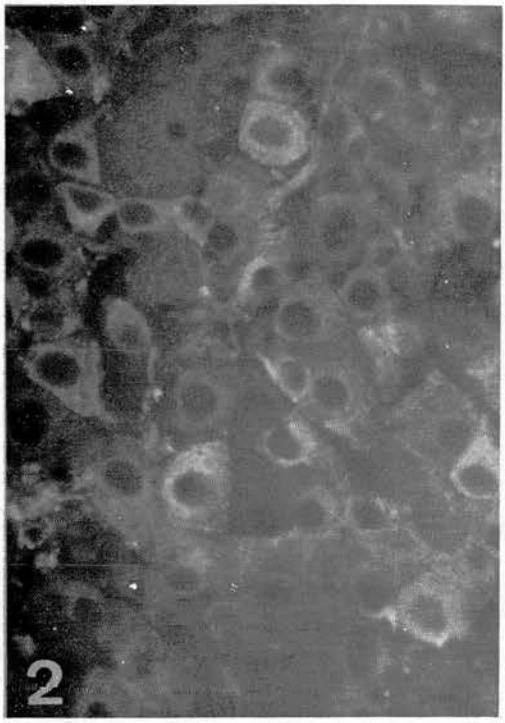
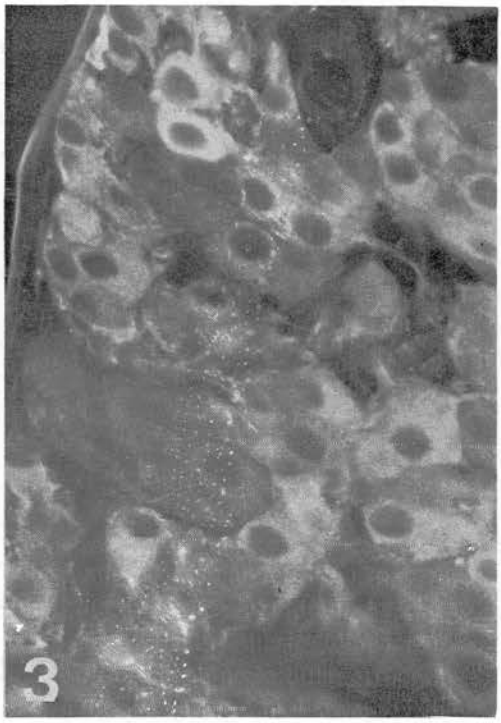


Fig. 1. Frequency–intensity distribution of cell bodies with 'negative' (−), 'moderate' (+) and 'intense' (++) fluorescence in the control and hydrocortisone-treated 5-day-old rats. The intensity of 200 nerve cells in each ganglion was visually estimated. The columns give the mean of 3 animals. Vertical bars show the standard error of the means. Ordinate: number of cells.





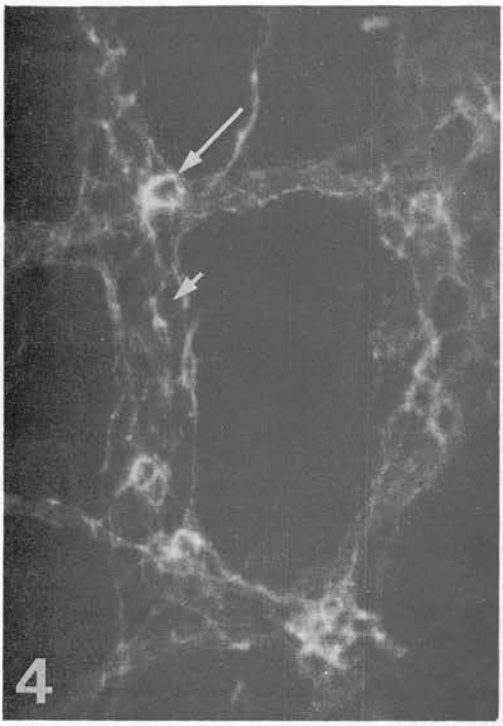
Figs. 2 and 3. Amine fluorescence in freeze-dried sections of the superior cervical ganglion of 5-day-old rats. × 362.
Fig. 2. Control rat. The nerve cell bodies show variable intensity of fluorescence. Few of them show an intense fluorescence.
Fig. 3. Hydrocortisone-treated rat. A larger number of cells show intense fluorescence.

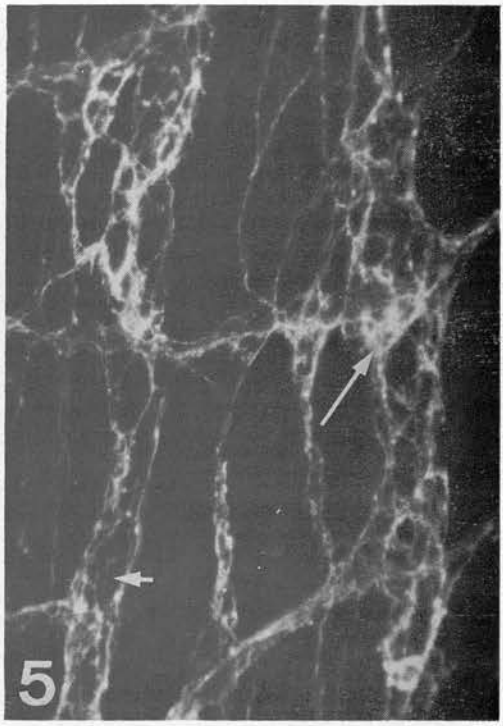
Intestine

In the 5-day-old control rats, the Auerbach plexus of the small intestine appeared as previously described[9]. The plexus was outlined by fibers of moderate fluorescence without prominent varicosities. In the nodes of the plexus, the myenteric ganglia, some varicose adrenergic fibers formed more intensely fluorescent pericellular structures (Fig. 4, long arrow). The non-fluorescent nerve cell bodies of the ganglia were visible as dark oval shapes outlined by fluorescent fibers (short arrows).

In the hydrocortisone-treated 5-day-old rats, Auerbach's plexus appeared more intensely fluorescent. All the fluorescent fibers were more clearly delineated than those of control preparations. The pericellular terminals were likewise more numerous in Auerbach's plexus of the hydrocortisone-treated animals.

The results of the photometric measurements of the fluorescence intensity are illustrated in Table I, which shows the mean exposure times of 5 randomly selected nodes of the plexus and the standard errors of the mean. In each animal, the fluorescence varied little between the ganglia measured. The effect of hydrocortisone was studied by comparing the intensity values of the control means and those of the





Figs. 4 and 5. Air-dried stretch preparation from 5-day-old control and hydrocortisone-treated rats. The exposure time and the developing time of the negatives and of the prints are the same in both figures. × 230.

Fig. 4. Myenteric plexus in the ileum of a control rat. In the ganglia of the plexus are visible fluorescent pericellular terminals.

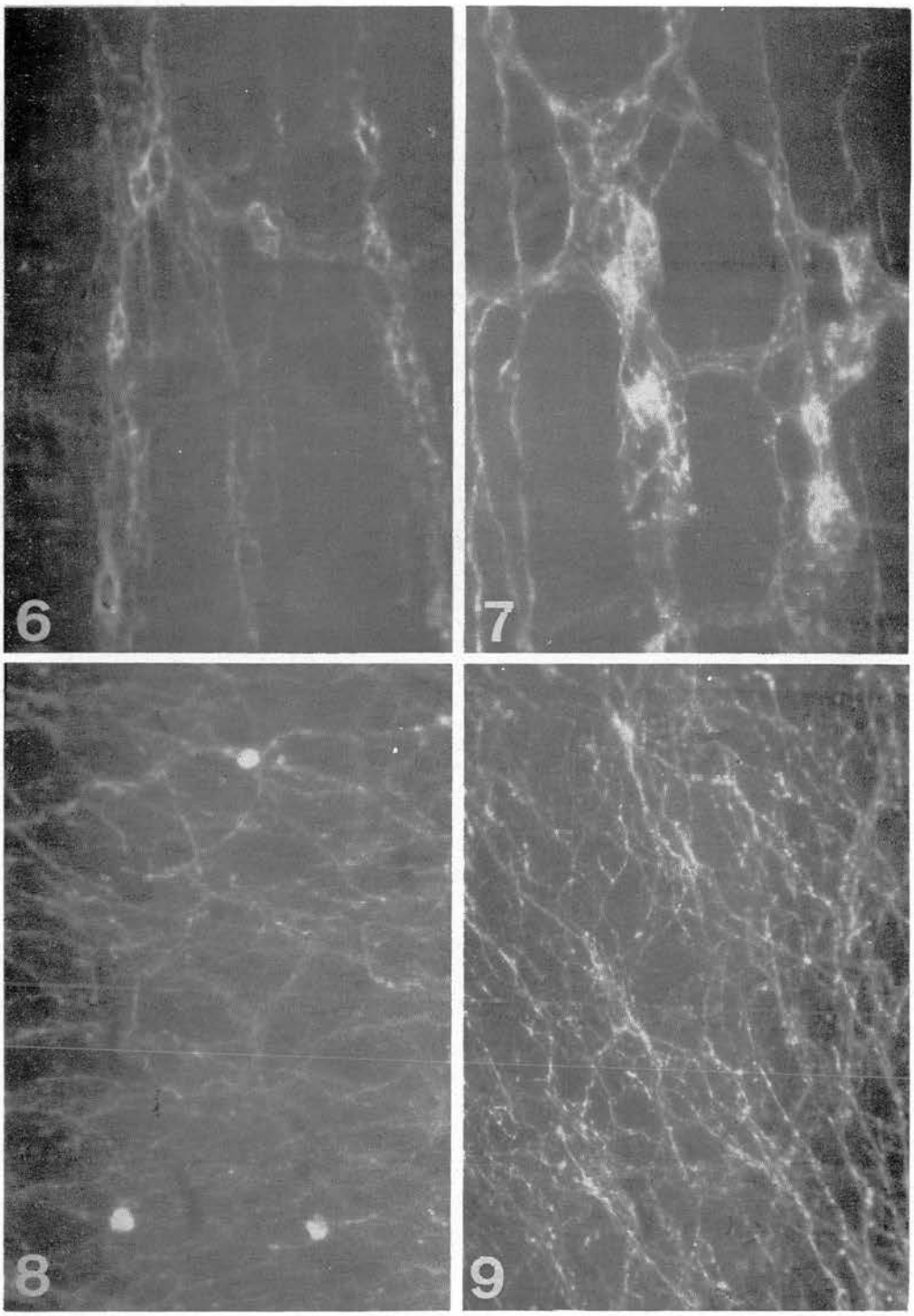
Fig. 5. Myenteric plexus in the ileum of a hydrocortisone-treated rat. The intensity of the fluorescent fibers is higher than in the control.

TABLE I

PHOTOMETRICALLY MEASURED FLUORESCENCE INTENSITIES OF THE ADRENERGIC NERVE FIBERS IN THE AUERBACH PLEXUS

Group	*Animal no.*	*Time of exposure (sec)* (mean of 5 fields ± S.E.)*
Controls	1	13.8 ± 0.58
	2	10.4 ± 0.24
	3	9.6 ± 0.24
	4	13.2 ± 0.37
Experimental	5	7.0 ± 0.31
	6	7.4 ± 0.24
	7	7.4 ± 0.24
	8	5.8 ± 0.20

* Under the conditions of the present study the time of exposure is inversely proportional to the light intensity measured. For details of the method see a previous report[3].


6
7
8
9

injected rats, using Student's *t*-test. A statistically highly significant difference ($P < 0.005$) was thus observed.

The increase in the fluorescence intensity after hydrocortisone treatment was still present 10 days after the cessation of the injections. In double-blind tests, each of the three authors independently detected a visually significant difference between 4 controls and 4 hydrocortisone-treated rats. Figs. 6 and 7 illustrate the Auerbach's plexus of the ileum from a control rat and from a treated rat allowed to recover for 10 days.

Iris

The normal appearance of the adrenergic terminal plexus in the iris at the fifteenth day of postnatal life is shown in Fig. 8. The terminal plexus of the iris showed marked differences in the hydrocortisone-treated rats even 10 days after cessation of treatment (Fig. 9) and these differences were shown to be significant by double-blind estimation independently by each of the three authors. Photometric measurement of the fluorescence intensity showed a statistically significant difference ($P < 0.01$) between the controls and the treated irides (Table II). Both the fluorescence intensity

TABLE II

PHOTOMETRICALLY MEASURED FLUORESCENCE INTENSITIES OF THE ADRENERGIC NERVE FIBERS IN THE IRIS

Group	*Animal no.*	*Time of exposure (sec)* (mean of 10 fields ± S.E.)*
Controls	1	8.5 ± 0.16
	2	9.2 ± 0.29
	3	10.0 ± 0.33
Experimental	4	7.4 ± 0.40
	5	6.1 ± 0.80
	6	7.2 ± 0.35
	7	6.1 ± 0.17

* Under the conditions of the present study the time of exposure is inversely proportional to the light intensity measured. For details of the method see a previous report[3].

Figs. 6–9. Air-dried stretch preparations from 15-day-old control and hydrocortisone-treated rats. × 226.

Fig. 6. Myenteric plexus in the ileum of a control rat. In the ganglia of the plexus some brightly fluorescent pericellular structures are visible. The photograph is underexposed since the exposure time and the developing time of the negative and the print are the same as those in Fig. 7.

Fig. 7. Myenteric plexus in the ileum of a hydrocortisone-treated rat. The number and the intensity of the fluorescent fibers and of the intensely fluorescent pericellular structures are increased.

Fig. 8. Iris of a control rat. The network of fluorescent fibers appears underexposed since the exposure time and developing time of the negative and the print are identical with those in Fig. 9. The brightly fluorescent spots are mast cells.

Fig. 9. Iris of a hydrocortisone-treated rat. The network of the fluorescent fibers appears denser and the single fibers are more intensely fluorescent.

of the fibers and the number of fluorescent fibers visible in a unit area were increased by hydrocortisone treatment.

DISCUSSION

The present work shows that hydrocortisone has a marked effect on the formaldehyde-induced fluorescence of the sympathetic nerve cell bodies and the adrenergic terminals of the newborn rat. A marked increase was observed in the fluorescence intensity of many cell bodies in the superior cervical ganglion. The fluorescence intensity and the number of fluorescent adrenergic terminals were also increased in the iris and in the myenteric plexus of the small intestine.

The question arises as to whether hydrocortisone affects the growth and differentiation of the adrenergic neurones or whether hydrocortisone affects some mechanisms which regulate the level of intraneuronal catecholamines, or whether it affects both.

An observation by Korochkin and Korochkina[10] tends to support the former alternative. They observed an increase in the size of the cell bodies of newborn sympathetic ganglia induced by hydrocortisone; this effect was not observed in the non-adrenergic ganglion cells of the intestinal plexuses. They also found that the cells of maturing sympathetic ganglia, but not those of the intestinal plexuses, show an accelerated protein synthesis after hydrocortisone treatment. The hydrocortisone-induced changes in the cell size and the protein synthesis decreased after the first days of postnatal life[10], as does the growth-promoting effect induced by the nerve growth factor[1] and the effect of hydrocortisone on the extra-adrenal chromaffin[11] and non-chromaffin[7] fluorescent cells.

On the other hand, hydrocortisone may cause an increase in the catecholamine concentration by inhibiting the activity of monoamine oxidase[15]. Inhibition of intraneuronal monoamine oxidase would result in an increase in the noradrenaline concentration in the cytoplasm of the adrenergic axons, which would then show a more intense fluorescence[12]. Since the fluorescence of the varicosities of the adrenergic fibers but not the fluorescence of the intervaricose segments increases following hydrocortisone treatment, the latter hypothesis appears less likely than the former.

Glucocorticoids are known to induce the enzyme phenylethanolamine-N-methyltransferase in the adrenal medulla and in the para-aortic chromaffin bodies[17], as well as in the sympathetic ganglia[2]. However, no evidence for an effect on tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) activities in the adrenal gland by glucocorticoids has been found[13,20,21]. However, the possibility still exists that hydrocortisone may induce the synthesis of TH and DBH in developing sympathetic neurons.

Chemical studies on the effects of hydrocortisone on TH and DBH of the nerve cells of sympathetic ganglia might be made difficult because of the presence of the small intensely fluorescent cells, which react markedly to this hormone[2,7]. It seems clear that glucocorticoids can affect the development of cells originating from the neural crest, including the developing sympathetic neurons. However, further work is

necessary to reveal whether glucocorticoids are involved in the control of development and maturation of the sympathetic nervous system.

ACKNOWLEDGEMENTS

The authors are very grateful to Dr. Daniela Costa for invaluable assistance in all stages of the study.

This work has been supported by grants from the Sigrid Jusélius Foundation (to O.E.) and Emil Aaltonen Foundation (to L.E.).

REFERENCES

1 ANGELETTI, P. U., LEVI-MONTALCINI, R., KETTLER, R., AND THOENEN, H., Comparative studies on the effect of the nerve growth factor on sympathetic ganglia and adrenal medulla in newborn rats, *Brain Research*, 44 (1972) 197–206.
2 CIARANELLO, R. D., JACOBOWITZ, D., AND AXELROD, J., Effect of dexamethasone in phenylethanolamine-N-methyltransferase in chromaffin tissue of the neonatal rat, *J. Neurochem.*, 20 (1973) 799–805.
3 COSTA, M., AND ERÄNKÖ, O., Histochemical correlates of cold-induced trans-synaptic induction in the rat superior cervical ganglion, *Histochem. J.*, 5 (1973) in press.
4 ERÄNKÖ, O., The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence, *J. roy. micr. Soc.*, 87 (1967) 259–276.
5 ERÄNKÖ, L., Postnatal development of histochemically demonstrable catecholamines in the superior cervical ganglion of the rat, *Histochem. J.*, 4 (1972) 225–236.
6 ERÄNKÖ, O., AND ERÄNKÖ, L., Intensely fluorescent granule-containing cells in the sympathetic ganglion of the rat. In O. ERÄNKÖ (Ed.), *Histochemistry of Nervous Transmission, Progress in Brain Research, Vol. 34*, Elsevier, Amsterdam, 1971, pp. 34–51.
7 ERÄNKÖ, L., AND ERÄNKÖ, O., Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra medullary chromaffin tissue, *Acta physiol. scand.*, 84 (1972) 125–133.
8 ERÄNKÖ, O., LEMPINEN, M., AND RÄISÄNEN, L., Adrenaline and noradrenaline in the organ of Zuckerkandl and adrenals of newborn rats with hydrocortisone, *Acta physiol. scand.*, 66 (1966) 253–254.
9 GABELLA, G., AND COSTA, M., Distribution of adrenergic fibres in the small intestine of the newborn guinea pig, *Panminerva med.*, 2 (1969) 407–409.
10 KOROCHKIN, L. I., AND KOROCHKINA, L. S., Hormonal influence on the differentiation of nerve cells of sympathetic and parasympathetic nervous system, *Z. mikr.-anat. Forsch.*, 82 (1970) 293–321.
11 LEMPINEN, M., Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it, *Acta physiol. scand.*, 62, Suppl. 231 (1964) 54.
12 MALMFORS, T., Studies on adrenergic nerves, *Acta physiol. scand.*, 64, Suppl. 248 (1965) 51.
13 MUELLER, R. A., THOENEN, H., AND AXELROD, J., Effect of pituitary and ACTH on the maintenance of basal tyrosine hydroxylase activity in the rat adrenal gland, *Endocrinology*, 86 (1970) 751–755.
14 OLSON, L., AND MALMFORS, T., Growth characteristics of adrenergic nerves in the adult rat, *Acta physiol. scand.*, 80, Suppl. 348 (1970) 66.
15 PARVEZ, H., AND PARVEZ, S., The regulation of monoamine oxidase activity by adrenal cortical steroids, *Acta endocr. (Kbh.)*, 73 (1973) 509–517.
16 PLOEM, J. S., The microscopic differentiation of the colour of formaldehyde induced fluorescence. In O. ERÄNKÖ (Ed.), *Histochemistry of Nervous Transmission, Progress in Brain Research, Vol. 34*, Elsevier, Amsterdam, 1971, pp. 27–38.
17 POHORECKY, C. A., AND WURTMAN, R. J., Adrenocortical control of epinephrine synthesis, *Pharmacol. Rev.*, 23 (1971) 1–35.
18 ROFFI, J., Influence de la corticosurrénale sur le contenu en adrénaline et noradrénaline de la surrénale, chez le foetus de Rat, *C.R. Acad. Sci. (Paris)*, 260 (1965) 1267–1270.

19 ROFFI, J., ET MARGOLIS, F., Synthèse d'adrénaline dans le tissu chromaffine extrasurrénalien chez le Rat nouveau-né, sous l'effet de l'hydrocortisone et de la corticostimuline, *C.R. Acad. Sci. (Paris)*, 263 (1966) 1496–1499.
20 WEINSCHILBOUM, R. M., AND AXELROD, J., Dopamine-β-hydroxylase activity in the rat after hypophysectomy, *Endocrinology*, 87 (1970) 894–899.
21 WURTMAN, R. J., AND AXELROD, J., Control of enzymatic synthesis of adrenaline in the adrenal medulla by adrenal cortical steroids, *J. biol. Chem.*, 241 (1971) 2301–2305.